ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL CANCER INSTITUTE
Fiscal Year 1981
Part II-B

U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service National Institutes of Health

ANNUAL REPORT

OF

PROGRAM ACTIVITIES

NATIONAL CANCER INSTITUTE (U.S.)

Fiscal Year 1981

Part II-B

Division of Cancer Biology and Diagnosis

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NATIONAL CANCER INSTITUTE

ANNUAL REPORT

October 1, 1980 through September 30, 1981

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DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE

SUMMARY REPORT OF THE DIRECTOR

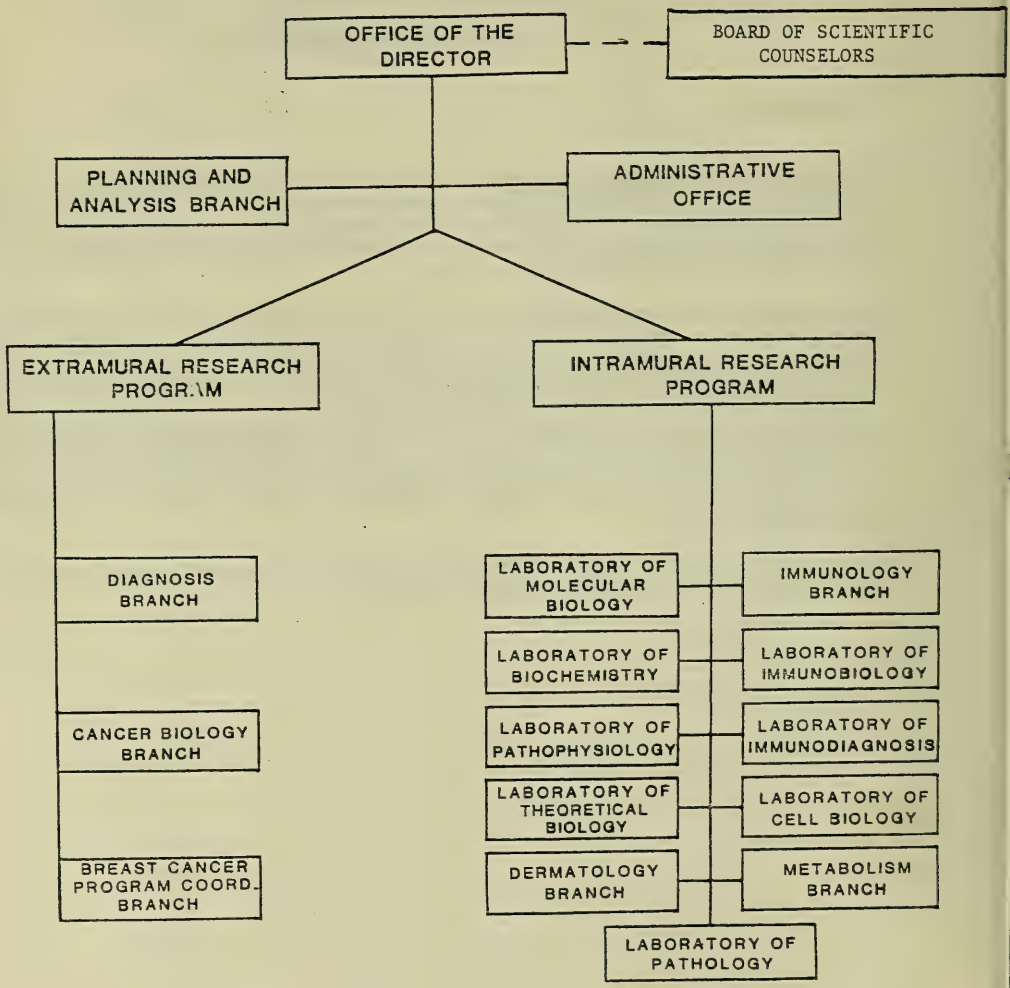
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INTRODUCTION

The Division of Cancer Biology and Diagnosis consists of both intramural and extramural research programs in the biology and diagnosis of cancer. The Division also coordinates the activities of the Breast Cancer Task Force and the Lung Cancer Screening Program. A close coordination is maintained with other divisions of NCI in areas of common interest.

The intramural research program consists of three broad areas: cancer biology, immunology and clinical research. A group of laboratories performing basic research in cancer biology includes the Laboratories of Biochemistry, Pathophysiology, Molecular Biology and Mathematical Biology. The Immunology Program includes work in the Immunology Branch and the Laboratories of Immunobiology, Immunodiagnosis and Cell Biology. Three clinical branches work in the fields of Dermatology, Metabolism, and Pathology. A fourth organizational element, under the Associate Director for Extramural Research Program, manages the four major extramural programs: Tumor Biology, Immunology, Cancer Diagnosis, and Breast Cancer Task Force.

The report of the Extramural Program is given in the second volume, which includes both contract and grant funded research.


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METABOLISM BRANCH

CANCER BIOLOGY

This report will cover three parts: Cancer Biology (Laboratories of Biochemistry, Molecular Biology, Mathematical Biology and Pathophysiology), Immunology (Immunology Branch, the Laboratories of Immunobiology, Immunodiagnosis, Cell Biology) and the clinical area covering the Dermatology Branch, Laboratory of Pathology and Metabolism Branch.

The research effort of the Laboratory of Biochemistry, directed by Dr. Maxine Singer, is focused on six areas: biochemistry of gene expression, biosynthesis, cellular regulation, developmental biochemistry, macromolecular interactions, nucleic acid enzymology and protein chemistry.

Investigators in the Biochemistry of Gene Expression Section have purified human glucocorticoid receptors sufficiently for use in the induction of antisera in rabbits. These sera will be characterized fully and used to assay receptors in both steroid-sensitive and -resistant cells. With enough receptor of 90% purity the group will seek to make monoclonal antibodies and to obtain partial amino acid sequence data. Dexamethasone mesylate was shown to be a covalent affinity ligand for glucocorticoid receptors. A nondissociable label for these proteins should assist greatly in their purification, in analysis of the structure of their ligand-binding sites, and in comparative analysis of receptors between species, tissues, and between sensitive and resistant cells. The rat growth hormone (GH) and prolactin (Prl) genes isolated in genomic clones are now being sequenced. A cell-free transcription system will be set up and parts of those genes will be used as templates. The rat and mouse GH gene content of L, GH_3 cell hybrids have been analyzed with eight restriction endonucleases. The intention is to microinject GH and Prl-specific and -nonspecific DNA's and L cell proteins into GH_3, L and hybrid cells to test the theory that L cell proteins are specifically shutting off GH and Prl production in the hybrids. Human leukemic (CEM) cell tumors have been established in nude mice and preliminary data indicate that an unorthodox steroid is a more effective antileukemic agent that are traditional glucocorticoids. A clone of CEM cells not lysed by but containing functional receptors for glucocorticoids has been found. It is hoped that this clone will provide information about the "post-receptor" steps in steroid action.

In the Biosynthesis Section, work continues on the study of the regulation of collagen synthesis in normal and transformed cells. It was previously found that sarcoma virus-transformed mouse fibroblasts have a marked increase in the synthesis of type III collagen relative to the usual type I and that specific phenotypic alteration persists at the non-permissive temperature. This suggests either that the src protein is not involved in the change or that it initiates the change but the reaction is essentially irreversible. Additional work involves the study of the enzymes and ancillary factors involved in DNA replication. The mechanism of E. coli DNA polymerase 1 large fragment has been examined using simplified reaction systems containing either $(dT)_{800}$ or $(dA)_{800}$ as template. It was found that termination at any given product chainlength was markedly increased by the presence of higher concentrations of the template primer complex. This, and results of other studies, suggests that the polymerase has more than one functional site for interaction with poly-

nucleotides. Other investigators have pursued studies of integrated intracisternal A-particle (IAP) genes in the mouse. They have shown that these genes, which are reiterated to the extent of 1000-2000 copies per genome in all strains of Mus musculus thus far examined, have structural aspects similar to the proviral DNA of type B and C retro-viruses. It was shown that sequences homologous to the IAP genes are endogenous to all mouse species tested and to several other rodent genera. A systematic survey has revealed several instances of IAP genes in proximity to other known mouse genes.

In the Cellular Regulation Section, recombinant DNA techniques were used to develop plasmid, phage, and bacterial vector systems which allow the isolation, characterization, and comparison of prokaryotic transcriptional regulatory signals. This system has been used to monitor the strengths of various promoters and the ability to regulate transcription from these signals when present on a high copy number plasmid. Coding information for a variety of prokaryotic and eukaryotic gene products is being fused into these systems. Previous work demonstrated that under the appropriate conditions prokaryotic genes could be expressed efficiently in eukaryotic cell-free systems. Recently investigators in this Section have demonstrated efficient expression of the E. coli galactokinase gene directly within mammalian cells. The ability to obtain expression of an easily asayable gene product from a structurally defined fusion vector now permits studies of: (1) functional complementation by the bacterial enzyme of primary cells isolated from human patients with galK-deficiency galactosemia or other galK-deficient mammalian cells; (2) transcriptional regulation in mammalian cells by fusing various eukaryotic promoters to the galK gene; and (3) translational regulation in mammalian cells by selectively altering the primary structure of the 5' non-translated portion of the transcript.

Additional studies have focused on the regulated expression of animal cell genes. Simian virus 40 (SV40) recombinants have been constructed which carry a variety of chromosomal eukaryotic genes. Experiments are now possible which allow examination of regulatory sequences, such as promoters and splicing sites, and also production of substantial quantities of useful gene products, such as human growth hormone and hepatitis B surface antigen, in cultured cells.

The Developmental Biochemistry Section continued to study the structure and expression of the genes for ribosomal RNA (rRNA) in Drosophila melanogaster. The nucleotide sequence were determined in several regions of these rRNA genes, including the region in which transcription terminates, and the boundaries between rRNA regions and insertions of type 1 and type 2. Data suggest that interrupted rRNA genes arose by the insertion of transposable elements into rDNA, leading to the inactivation of these genes. Additional investigators have studied gene expression during muscle development in the chicken. The proteins encoded in the most abundant mRNA class expressed in embryonic chick muscle have been identified. Certain isozymic forms of these proteins are expressed only in differentiated muscle, whereas all cells express the other forms of these protein. The muscle specific set of protein appears to be coordinately regulated, in that all these genes are switched on synchronously during differentiation. Using defined, cloned ds-cDNA probes for these genes, genomic sequences for some of these proteins have been isolated. The organization, structure, and regulation of the set of differentiation specific and the constitutive genes is being compared.

The Macromolecular Interactions Section, directed by Dr. Claude B. Klee, is concerned with the mechanism of regulation of cellular processes in eukaryotes by the Ca^{2+} receptor protein, calmodulin. The five different Ca^{2+} calmodulin conformers are being characterized further by limited proteolysis and fluorescent probes to facilitate study of the mechanism of the Ca^{2+}-dependent interactions of calmodulin with its several target proteins. A unique feature of calmodulin is its ability to interact with a large variety of different enzymes and proteins. Studies on structural relationships between the different calmodulin-dependent enzymes are now being pursued to characterize the molecular mechansim of these specific interactions. An interaction between calmodulin and the regulatory subunit of cAMP-dependent protein kinase in brain preparations has been demonstrated which results in an increased affinity of the protein kinase's catalytic subunit for its regulatory subunit. The multiple links observed between the two messenger systems provide a frame work upon which the intricacies of cellular responses to external stimuli can be built.

The Nucleic and Enzymology Section, is interested in the relation between the genomes of simian virus 40 and the African Green Monkey (AGM). This relation has been investigated using recombinant DNA techniques to isolate DNA segments related to SV40. A library of the AGM genome in λ bacteriophage was prepared. This library yielded 3 different AGM DNA segments that hybridized, to the region around the SV40 origin of replication. The ability of the AGM segments to function as origins of replication and as "promoter"-like elements is being investigated.

The structural organization of the highly repeated DNA of the African Green Monkey is also under study. One highly repeated DNA of AGM is largely contained within a cryptic satellite called α-component. The α-component sequences in single monkey chromosomes isolated by construction of somatic cell hybrids between AGM cells and mutant rodent cells is now being studied. One cloned cell line carrying a single monkey chromosome (with gene for thymidine kinase) has been obtained. Data suggests that this chromosome contains a family of α-component sequences markedly different from the entire set present in AGM. In another approach to studying α-component organization, a library of total AGM DNA in lambda phage was constructed in such a way as to select against the most abundant type of α-component organization, namely, long tandem repeats with no EcoRI site. Analysis of 16 clones has revealed that two subfamilies of AGM segments can be defined on the basis of common junction sequences.

Other investigators have used recombinant DNA technology to explore the organization and expression of two yeast genomes. One was found to code for a repressible acid phosphatase (APase) and the other for the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase. In both cases, several of the multiple copies were isolated.

The APase gene copies have been isolated and the structure of the APase genes and the mRNA map positions have been determined. One of the APase genes has been inserted into a yeast vector, and deletion mutations have been constructed to define the sequences required for regulation and expression of the APase gene.

In the Protein Chemistry Section, Mammalian hprt and tk genes have been transferred to eukaryotic cells by DNA-mediated gene transfer. These transformed cell lines are being used to clone the tk gene. Somatic cell hybridization has been

used to prepare a panel of 25 independent human/rodent hybrid cell lines segregating human chromosomes. The specific human chromosomes present in each line have been determined. DNA was isolated from each line, and gel blots were prepared after digestion with restriction endonucleases, followed by agarose gel electrophoresis. These blots have been used to map human immunoglobulin genes and MSV src genes to specific human chromosomes.

Investigators have developed a preparative nonlytic separation of $Lyt2^+$ and $Lyt2^-$ T cells, which has enabled them to examine directly the question of help for the generation of cytotoxic effector cells (CTL). It was found that the lymphokine "IL2" can substitute for $LYT2^-$ cells in providing help to splenic CTL precursors, resulting in the generation of CTL from highly purified $Lyt2^+$ responder spleen cells in primary mixed lymphocyte culture.

The inflammatory response in guinea pigs is being used as a model to study the nature of feedback regulators of granulocyte differentiation. It has been shown that 12-hour inflammatory (s-12) serum stimulates the mitotic activity and the glycosylation of granule components of bone-marrow granulocyte precursors in primary cell cultures. Additional data suggest that the mitotic- and the glycosylation-stimulating activities are associated with separate, nondialyzable serum components.

The transplantable granuloctyic leukemia (GL-13) previously developed in strain 13 guinea pigs is now in its 80th transplant generation. In one transplant line the leukemia has recently undergone a significant change making it a valuable model for studies of the blast crisis in human leukemia. Cytogenetic studies of the GS-13 show a chromosome defect in these cells, a terminal deletion in one of the long arms of a single chromosome resembling the Philadelphia chromosome-positive (Ph^{1+}) type of human chronic myelocytic leukemia.

The research effort of the Laboratory of Molecular Biology, directed by Dr. Ira Pastan, is focused on understanding the factors that control gene expression in animal and bacterial cells and on understanding of the biochemical basis for the abnormal behavior of cancer cells.

Transformation of fibroblasts by Rous sarcoma virus produces alterations in cell shape, growth and metabolism, by the action of a single gene *src* which codes for a 60,000 molecular weight protein. This transforming protein has been purified from Rous sarcoma virus induced tumors. Previous studies showed that it is concentrated at the plasma membrane of cells, indicating that the *src* protein generates a signal that instructs the nucleus to begin DNA synthesis and to alter the activity of some of its genes. Two of these genes are those for fibronectin and collagen, coding for diminished adhesion of these cells. The genes for collagen have been cloned in *E. coli* and are being used for studies of gene activity in normal and transformed cells. The mechanism by which fibronectin controls cell adhesion is also being investigated.

Studies are underway to isolate mutant cells which contain host mutations that affect the transformation process. Chinese hamster ovary (CHO) cells have been transformed with Rous sarcoma virus, and revertant cells (which have regained normal growth control and shape) have been isolated.

Overlapping genomic clones containing the entire gene for pro-alpha collagen have been isolated. Further studies have shown that, (1) much of the gene is made up of exons of an identical size of 54 base pairs, suggesting that the ancestral gene for all collagens was assembled by amplification of this single genetic unit and (2) there are three large mutually overlapping dyads of symmetry in the promoter region of the gene which could constitute binding sites for regulatory proteins.

Eucaryotic vectors, which function both in E. coli and in mammalian cells, have been constructed by recombinant DNA techniques. These vectors can be used to isolate and define eucaryotic genetic segments. A number of coding sequences from E. coli have been inserted into the vector pSV2. These genes have been amplified in E. coli and the DNA used to transform animal cells. Recent studies indicate that these genes can be expressed in animal cells. Analysis of the regulation of their expression is underway. Construction of these vectors offers, not only useful techniques for analysis of gene function, but perhaps also for the therapy of certain diseases.

Research efforts in the Molecular Cell Genetics Section have concentrated on the use of mutational and biochemical analysis of cultured mammalian cells to study some aspects of their behavior. Specifically, the mechanism of action of cyclic AMP and regulation of its metabolism, have been studied using CHO cells. Additional studies have shown that hormone-mediated functions of mammary epithelium require DNA synthesis, yet are independent of cytokinesis. The pathway of tranformation of mammary epithelium has been shown to be different from the pathway of MMTV-induced transformation.

Fibronectin is a major adhesive protein of fibroblasts and of other types of cells. Investigators in the Membrane Biochemistry Section have found that fibronectin has one structural domain of 27,000 daltons that binds to actin, and a domain of 50,000 daltons that binds to heparin. Studies have shown that fibronectin is present at the "close" adhesive contact regions of cells and not the "focal" adhesive sites, indicating that it has a role in cell movement and not in cell immobilization.

Cultured cells have on their surface, specific receptors for growth promoting substances, hormones, and other ligands. Receptor-ligand complexes are trapped in special coated regions of the membrane called clathrin-coated pits. They are then transferred to a "receptosome" which appears to bud off from the neck of a coated pit, as seen in the electron microscope. The function of the receptosome pathway is to internalize ligands and send them to specialized regions of the cell without allowing them to be transferred to lysosomes and destroyed. Studies indicate that the clustering of some ligand receptor complexes in coated pits is dependent upon the activity of a transglutaminase-like enzyme in the pits, and efforts to isolate this enzyme are underway.

The Biochemical Genetics and Developmental Genetics Sections have been studying molecular mechanisms of the switch from early to late transcription in bacteriophage lambda. They have found that the high level of transcription from the early $P_{R'}$ promoter prevents access of the transcription proteins to the phage later promoter $P_{R'}$, thus occluding the $P_{R'}$ activity. To study the regulation of the regulatory proteins, the rho gene has been cloned. Genes for adenylate and for cyclic AMP receptor protein (CRP) are being cloned. It has been shown that the level of Pho protein in E. coli is medulated by cyclic AMP and CRP.

The research effort of the Laboratory of Pathophysiology, directed by Dr. Pietro Gullino, has focused on several areas: pathophysiology of the mammany gland and tumors; biochemical changes related to onset and cessation of normal cell growth; function and control in eucariotic cells; freeze-fracture observation of normal and neoplastic cell membrances; effects of ionizing radiations on nucleic acids, proteins and bone marrow; total metabolism of cancer cachexia; and the search for antitumor agents by preparation of amino acid analogs.

Studies were undertaken to explain the events conductive to mammary tumor regression after removal of the ovaries. It was shown that oral administration of {^{3}H} DB cyclic AMP concentated twice as much radioactivity in the mammary tumor and mammary gland as did parenteral injection. In regressing tumors where cyclic AMP dependent protein kinase activity is enhanced, the activity of RNA polymerase II is decreased. The effect of DMBA treatment on the cyclic AMP system of the mammary gland is being studied. When DB cyclic AMP was given to the animals treated with DMPA, the number of mammary tumors was sharply reduced; the onset was delayed, and the changes in the cyclic AMP system were blocked. The preventive action of DB cyclic AMP on oncogenesis by DMBA is being evaluated.

The control of adenylate cyclase activity in normal and neoplastic cells is being investigated. An increase in activity was observed in crude NRK membrane preparations incubated in the presence of activated cholera toxin, GTP, ATP, NAD^+, when a protein a MW of approximately 13,000 was also present. The responsiveness of adenyl cyclase to hormones is lost when NRK cells are transformed by Kirsten or Maloney viruses; this is a consequence of a defect in the coupling of the receptor to the catalytic subunit of the cyclase.

Basal, GTP- and fluoride-stimulated adenylate cyclase showed a progressive increase when F9 line of teratocarcinoma cells were induced by retinoic acid to differentiate into parietal endoderm cells. Whether the modified responsiveness to hormones is due to alteration of the GTP coupling mechanism of the cyclase or to changes in the hormone receptor has yet to be established.

MCF-7 human mammary carcinoma lines produce tumors in nude mice, and estradiol increases tumor growth 5-6 fold. Estrogen treatment of nude mice was shown to depress NK cell activity while ovariectomy increased it suggesting an involvement of NK cells in the growth of MCF-7.

Looking for the evidence that hormone dependency in mammary carcinomas is related to the cell composition, it was observed that primary NMU tumors are ovarian dependent for growth, consist of both epithelium and myosepithelium, are estrogen receptor positive, synthesize and require type IV collagen for growth, and produce a sarcoma growth factor-like substance. The growth factor produced by NMU tumors in vivo and in vitro is being isolated and purified. A human prototype II collagen has been isolated from a human condrosarcoma group in nude athymic mice. A neutral protease has been extracted from the media of cultured metastatic cells and found to cleave specifically both chains of the type IV collagen. A new assay has been established to test for correlations between invasiveness and the metastasizing capacity of a cell population. A series of experiments has been undertaken to correlate the hypothesis that acquisition of angiogenic capacity by a cell population indicates an increased risk of neoplastic transformation. A project on tumor hyperthermia has demonstrated the extent of anisotropy of blood distribution with the tumor and the need to rely more on thermal diffusion than on thermal convection in hyperthermic treatment.

Cells from a mouse teratoma and mammary epithelium have been used in a set of experiments aimed at defining the role of promoters (TPA) on oncogenesis. TPA was found to inhibit neutrophil chemotaxis induced by formylated tripeptides. This effect is abolished by glucocorticoids, due to the presence of an inhibitor protein.

Prolactin binding to rodent liver membranes increased by 60% when hepatocyte were incubated with phospholipase A_2 on lysolecitin. To test the hypothesis that this was due to changes in PRL-receptor availability, the fluidity on the microsomal membrane was compared during PRL treatment. Prolactin receptors appear as the fluidity of the membrane increases, suggesting that specific prostaglandins modulate prolactin receptors in vivo via changes in the fluidity of the cellular membranes. This mechanism probably operates in tumors also.

Experiments have been carried out to assess whether events occurring during the development of the mammary gland can condition the future pathological history of the aging gland. Other experiments evaluate the role of T_3 and prolactin in mammary gland development and milk production. Synthesis and secretion of α-lactoalubumin were enhanced by T_3 in cultured mammary gland cells, and DNA synthesis was shown to be necessary for the initiaton of casein production.

A series of experiments designed to investigate the conditions controlling protein synthesis in lyphocytes are undeway. Work is progressing to test the hypothesis that changes in tRNA. Met concentrations may have a regulatory role in protein synthesis. The interaction between the surface protein stimulating T cells and protein synthesis is being evaluated.

A new "fracture-label" method has been developed which permits the study of distribution and partition of lectin binding sites. This method has been used to study the erythrocyte membrane, the structure of the prostate gland, the process of myelin formation and the spatial arrangement of membrane glycoproteins in the platelets.

The UV photolysis on tryptophan and tryptophan containing peptides in aerated aqueous solutions has been studied by e.s.r. and spin trapping techniques using T-nitrous-butane as Uspin-trap. U.V. irradiation of animo acids and peptides at room temperature has been studied with light of h 300 nm in the presence of dibenzoylperoxide, a compound used to treat facial acne and now believed to promote carcinogenesis. These studies point to the possibility of deleterious effect when topical application of benzoylproxide is followed by exposure to sunlight.

The research activities of the Laboratory of Mathematical Biology (LMB), directed by Dr. Mones Berman, fall into several broad areas: membrane biophysics, immunology, macromolecular configurations, kinetics of metabolic systems and computational and modeling methodology. Most of the work is theoretical, but experiments are also carried out in the laboratory.

The assembly, topology and conformation of proteins in membranes has been investigated by the physical-chemical approach, using lipid model membranes. The dynamics and organization of membrane lipids are being studied since interfaces between domains of lipids with different packing might serve as sites for insertion of membrane proteins. To understand the mechanism whereby secreted proteins

are translocated across the endoplasmic reticulum membrane, ovalbumin is being studied. When ovalbumin is denatured in vitro it does not return to its native form upon renaturation but assumes a new form called OAR. OAR is being used as possible ligand to identify protein translocating sites on the rough endoplasmic reticulum. The finding that OAR is immunologically distinct from native ovalbumin will enable the determination of which form is initially synthesized on ribosomes in the presence and absence of membranes.

Collaborative studies continue on the development and application of mathematical techniques for describing receptor clustering on the plasma membrane. The results were incorporated into a theory of the response of sensitized basophils and mast cells. Experimental applications of the theory of cell surface events were extended to IgG complexes interacting with Fc receptors on macrophages. This work is relevant to immune complex effector mechanisms, and may also provide insight into the analysis of data on a number of other systems, such as those involving binding of hormones or neurotransmitters to cell surface receptors.

Another area of research within the laboratory concerns biological macromolecules and their properties. Stabilities of macromolecular conformations are determined by their interatomic interactions. The relative importance of various classes of interactions is being assessed in detail. Short and medium range interactions appear to determine most of the regular secondary structures such as α-helices and β-strands. Several simple models of long range intramolecular interactions have been formulated to facilitate investigations of protein folding pathways. More detailed calculations of conformational energies, requiring evaluation of all interatomic distances, are feasible for protein fragments. Two new binding sites for lysozyme have been postulated, and these sites are being tested experimentally with monoclonal antibodies specific to the binding site. Similar calculations are being applied to the variable regions of the antigen combining site of myeloma immunoglobulins.

Modeling of the endocrine systems has continued with emphasis on lipoproteins, the glucose-insulin system and receptors. The model for lipoproteins has been extended by studying direct triglycerides synthesis pathways for Low Density Lipoproteins and High Density Liporproteins. The major effort in the glucose-insulin system involved the integration of the glucose and gluconeogenic precursors subsystems into a single, physiologically oriented model. Studies of insulin secretion and its control have also been resumed.

Research in the Immunology Branch has focused on the following areas: 1) regulation and control of immune responses; 2) structure and function of cell surface molecules; 3) transplantation biology; and 4) tumor immunology, including clinical studies in immunotherapy.

Work in the first area has been directed at studies of the generation and regulation of T cell dependent responses to both conventional and allo-antigens, and at the mechanisms of interaction among T cells, B cells and accessory cell populations. Studies have characterized the T cell subpopulations involved in the generation and regulation of cytotoxic T lymphocytes directed toward H-2 different stimulators.

Collaborative studies concerning genetic control of the immune response to staphylococcal nuclease have also been carried out. The in vitro antibody

response to TNP-nuclease has been shown to be under the control of immune response Ir genes the and the function of such genes is expressed at least in part at the level of antigen-presenting or accessory cells. The function of Ia antigens in Ir gene expression has been further evaluated in both T cell dependent antibody responses and antigen-specific T cell proliferation. Monoclonal anti-Ia reagents, including those specific for gene complementation products of I-A and I-E subregions, inhibit in antigen-specific fashion the T cell recognition of antigen, consistent with a direct or steric relationship between Ia antigens and Ir gene products.

Studies of the genetic control of the immune response to nuclease, have examined the in vivo effects of treatment with anti-idiotypic antibodies. Injection of pig anti-idiotypic antibodies into virgin mice has been found to lead to an increase in idiotype levels in the serum of these mice. This idiotype was found predominantly on immunoglobulin molecules without antigen binding activity for nuclease. Similar treatment of nude mice did not lead to idiotype expression in serum, suggesting that the effect might involve T cells. Additional studies were performed to examine the expression and functional involvement of idiotype on T cells in a system of T cell dependent antibody responses to TNP-nuclease. Antibody responses to TNP-nuclease were inhibited by anti-idiotype, in vitro, and this effect was mediated by an effect upon helper T cell function, supporting the concept that idiotypic determinants expressed by T cells are important to the function of those cells. Anti-idiotype administered in vivo was shown to result in the generation of idiotype-bearing nuclease-specific primed helper T cells, demonstrating the ability of anti-idiotype to trigger these cells in the absence of antigen.

Further investigations have been directed towards the study of the genetic control of immune cell interactions among macrophages, T cells and B cells. It has been shown by anti-Ia inhibition experiments that the I region encoded restricting elements, which helper T cells recognize, are the Ia antigens macrophage express. The experiments provide strong evidence that the Ia antigens are the products of Ir genes.

Other experiments have shown that developmentally distinct B cell subpopulations are activated by helper T cells in genetically distinct ways. The activation by T cells of Lyb5-B cells is MHC restricted while the activation of Lyb5$^+$ B cells is not. Further functional studies of B cell subpopulations are underway. These experiments suggest a resolution to a long standing controversy in cellular immunology over the question whether or not T-B interactions are genetically restricted.

Other studies of the regulation and control of immune responses have been directed toward understanding the role of the major histocompatibility complex in regulation and restriction of T cell mediated and effected immune responses against chemical haptens and infectious viruses and toward the genetic and mechanistic aspects of natural resistance to graft-vs-host reactions. A new series of hapten-self-specific CTL responses have been developed in the mouse. Results suggest that CTL effector cell specificity is strongly influenced by the haptenic portion of the foreign antigen-self immunogenic complex, whereas the pattern of Ir gene control is greatly and possibly exclusively determined by the self determinant recognized.

It is now clear that several distinct membrane receptor interactions take place upon binding of ligand to receptors. The involvement of the Fc portion of the IgG antibody in each of these interactions provides substantive evidence for a central role for this receptor in B lymphocyte function.

Additional research is directed towards understanding the interactions which may take place on cell surfaces via idiotype-anti-idiotype recognition. A system has been developed which allows in vitro production of antibody responses to an antigen (T,G)-A--L. The characteristics of this response in vitro are identical to those of in vivo responses, and idiotype can be detected on such antibody. Other investigators are studying the molecular and cellular basis of the interactions of immunoglobulins with immune effector systems, and the relationship of antigenic recognition to these interactions. Techniques have been developed for measuring the binding of model immune complexes to Fc receptors on cells.

Studies in the Transplantation Biology Section have been directed toward understanding the structure and functions of the products of major histocompatibility complex, and manipulations of the immune response to these products. A large number of hybridoma cell lines producing antibodies have been used to further subdivide products of the MHC. Anti-idiotypic antibodies against these hybridomas have been produced and the effects of such anti-idiotypic reagents on in vitro and in vivo parameters of histo-compatability have been examined. These anti-idiotypic antibodies may provide an approach to modification of the immune response to MHC antigens.

Transplantation studies using the miniature swine model have continued. Two new recombinants within the MHC have been detected within the mimiature swine herd. Both recombinants involve separation of the MLC stimulatory locus (SLA-D) from the serologic loci (SLA-ABC). Transplantation studies aimed at determining the relative importance of individual MHC loci are now in progress.

The nature of the alloreactive T cell repertoire has been examined, suggesting that the T cell repertoire for alloantigens, like that for conventional antigens, may be both MHC restricted and environmentally modified. Additional work showed that the self-recognition repertoire of thymocytes is absolutely determined by radiation-resistant thymic elements, and that T cell precursors are specifically tolerized by their pre-thymic environment. This suggests that T cell precursors express their receptors for allogeneic determinants prior to their entry into the thymus. This has important implications for our understanding of how the T cell receptor repertoire is generated.

Other laboratory interests focus on understanding the nature of human T cell recognition and activation, with a particular emphasis on the genetics of the human major histocompatibility complex (HLA). A new HLA gene, designated "secondary B cell" (SB) gene was defined. The relevance of these new SB markers to multiple sclerosis and dermatitis herpetiformis is under investigation.

Studies continue on the organization of genes encoding the major histocompatibility complex and mechanisms controlling the expression of these genes. Recombinant DNA technology has been used to isolate genomic fragments containing MHC genes of both mouse and pig. Studies on the regulation of gene expression have been directed at isolating and characterizing RNA species encoding MHC antigens. In both the mouse and miniature swine, mRNA species encoding MHC

antigens have been identified. A novel RNA species, containing sequences homologous to MHC sequences has been found in the miniature swine. A coupled system of in vitro translation and processing is being developed to study the role of post--translational processing in the regulation of MHC antigen expression.

A major research effort in tumor immunology has been directed toward identification of factors which influence host cytotoxic cell responses against syngeneic tumors. A fully syngeneic in vitro system for generating primary mouse cytotoxic-cell responses detected by 4-hour chromium release assays with MCA-induced tumor target cells has been established. The effector cells were shown to be a type of natural killer (NK) cell. However, in vitro generation of these anti-tumor NK cells and their activity differ significantly from that which occurs naturally in vivo and the in vitro stimulation appears to result in a subpopulation or different population of NK cells compared to those occurring naturally in vivo. Additional studies of the in vitro anti-tumor cytotoxic cell responses indicate that they may be controlled by multiple non-H-2 genes.

A controlled, randomized comparison of immunotherapy to chemotherapy in Stage I and Stage II malignant melanoma has continued. Patients have been randomly assigned to receive treatment either with methyl CCNU, BCG alone, BCG plus allogeneic tissue-culture-grown vaccine, or no treatment. A total of 181 patients have entered the trial, and patient accrual has been terminated.

The interest of the investigators in the Macromolecular Biology Section is the elucidation of macromolecular changes on the surface of mammlian cells, and how such changes relate to certain normal cell surface functions pertinent to cell division and to the appearance of cellular tumorigenicity.

A primary thrust of this laboratory has been to identify, isolate and characterize an SV40 induced cellular phosphoprotein Primary cells prepared from mid-gestation mouse, rat and hamster embryos expressed protein constitutively without SV40 infection. The 2D tryptic map showed that this protein is similar to SV40 induced 55K protein and is conserved evolutionarily in both embryonic and in SV40 transformed cells. The amount of the protein in the embryo was found to be half that of SV40 transformed cells and its presence correlated with the age of the embryo. The biochemical nature and possible functions of this protein are also being studied. Experiments suggest that it may be involved in control reactions pertaining to DNA replication and cell growth and division. The expression of the protein, however, does not correlate with tumorigenic transformation in established mouse cells.

Studies continue on the characterization of the well pedigreed families of normal mouse cells, or cells transformed spontaneously or with SV40. It was found that spontaneous transformation is sufficient to explain the acquisition of cellular tumorigenicity of the SV40 transformed mouse fibroblasts. There is no binding correlation between cell growth in viscous medium, of tumorigenicity in vivo in both syngeneic and in nude mouse and of the SV40 expression in the mouse fibroblast cell families we have investigated. This confirms the previous observation that the phenotypic changes pertaining to in vivo properties of cells attributed to SV40 early gene coded T antigen are predominantly expressed as cell surface antigens, facilitating immunologic recognition and rejection of the cells in the immmunologically competent syngeneic mouse.

The research activities of the Laboratory of Immunodiagnosis, directed by Dr. Ronald Herberman, can be divided into four main areas: role of natural and induced effector and suppressor cells in resistance against cancer; cell-mediated immunity against tumor associated and other antigens; immunochemical studies of tumor associated antigens; and application of immunologic procedures to clinical problems of cancer patients. The main objective of all these studies is the application of the research information to the diagnosis of cancer and the monitoring of disease in tumor bearing individuals.

A major emphasis has been on the study of natural killer (NK) cells; their characteristics, and their in vivo role in resistance against tumor growth. Highly purified populations of human and rat large granular lymphocytes (LGL) have been isolated. This small subpopulation of cells has been shown to be responsible for NK and antibody-dependent cell-mediated cytotoxic activity. LGL have been extensively characterized and shown to share features with T-cells and monocytes. LGL were found to produce interferon (IF) in response to a wide variety of stimuli.

Appreciable progress has been made regarding the cytotoxic, accessory and suppressor functions of macrophages and monocytes, and the processes involved in their activation. There has been special emphasis on the production of somatic cell hybrids, to develop of B cell hybrids making monoclonal antibodies to subpopulations of human and mouse lymphoid cells and to human lung tumor associated antigens, and T hybrids secreting lymphokines.

Considerable progress has been made in the purification of a human lung tumor associated antigen and in the development of a practical radioimmunoassay which can detect elevated levels of this antigen in the serum and urine of lung cancer patients. Investigators in this laboratory have also developed the ability to monitor in detail and in a standardized manner the immunologic reactivity of cancer patients receiving various biologic response modifiers. This is currently being utilized to study the immunologic effects induced by purified recominant leukocyte IF.

Studies on the role of NK cells for resistance against cancer have focused on the characteristics of NK cells and the possible mechanisms for their cytotoxic activity, on factors and agents affecting the levels of cytotoxic activity and the mechanisms for these effects, on production of IF by NK cells, and the relationship of NK activity to in vivo resistance against tumor growth.

A major advance in the characterization of human NK cells has come from the finding of their close association with LGL. A considerable portion of these LGL form lytic conjugates with NK-susceptible targets. Because of the ability to obtain highly purified human and rat LGL and their strong association with human NK cells, investigators have been able to perform detailed phenotypic studies with these effector cells and compare them to the phenotype of typical small, mature T lymphocytes. As in the human studies, the rat LGL were found to be an antigenically distinct population of cells which share some characteristics with monocytes, T cells, and granulocytes.

Efforts have been made to determine the mechanism of cytotoxicity by NK cells. Substantial evidence was obtained for a role of serine proteases. Transmethylation and phospholipase A_2 activity were found to increase during the NK

cell-target cell interaction. Studies using purified mononuclear cell fractions enriched for LGL, showed that these cells demonostrated the same properties of transmethylation and phospholipase A_2 activation.

IF has been shown to have a variety of effects on immune reactivity, including the ability to rapidly augment cell-mediated cytotoxic responses, such as the reactivity of NK cells, and macrophages, or monocytes. Some of these effector mechanisms may have in vivo importance in the resistance against tumor growth or against infections by various microbial agents. Studies have demonstrated substantial quantitative differences in the ability of the various species of human leukocyte IF to significantly augment levels of cell-mediated functions. Such results should have a significant impact in selecting IF species for appropriate clinical trials.

In addition to natural leukocyte IF's, the newly available bacterial-produced homogeneous leukocyte IF has been examined for its augmentation of cytotoxic activity of NK cells and monocytes. As with the purified species of leukocyte IF, its relative potency was tested at various antiviral units. In parallel experiments, recombinant IF and natural IF were found to have similar activities, in providing a further basis for optimism regarding its potential usefulness in clinical trials.

Detailed analysis of treatment of human LGL with IF, has indicated that it has multiple effects on NK cells depending on the target cell tested. IF increases phospholipase A_2 activity principally on substrate derived from the transmethylation pathway.

Several regulatory mechanisms have been identified which can depress the levels of NK activity. Both spontaneous and IF-boosted NK activity were inhibited by the addition of macrophages. Since there is low NK activity in the peritoneal cavity, this may represent one mechanism by which NK activity is naturally suppressed. Short-term incubation of human peripheral blood mononuclear cells in medium lacking human serum was shown to increase NK activity. The serum-mediated effect appeared attributable to the degree of binding of cytophilic monomeric IgG to the cells, suggesting that this mechanism may be involved in negative regulation of NK activity in vivo.

The NK cells appear to be able to respond to certain stimuli by producing IFs that in turn can augment their reactivity. NK cells may have a broader range of biologic effects than were initially appreciated, with a potential for directly producing an antiviral protein and for affecting the activity of other immune effector activities responsive to IF.

Investigators continued to assess in vivo NK activity by measurement of the elimination from the lungs of intravenously inoculated radiolabeled tumor cell lines. Intravenous transfer of normal spleen cells or bone marrow cells can restore the depressed in vivo resistance induced by cyclophosphamide. The spleen cells required for this in vivo transfer of resistance have the characteristics of NK cells. After depression of NK activity in nude mice, purified populations of human LGL, which have been shown to be highly enriched in NK activity, were transferred and the mice were then challenged with radiolabeled human or mouse tumor cells. Such transfer of human LGL was able to increase the clearance from the lungs of the human NK-sensitive tumor cells, but had no effect on clearance of mouse tumor cells that are resistant to human NK activity.

Similar studies have been performed with transfer of mouse and human cultured lymphoid cells (CLC) that have NK-like cytotoxic activity. Systemic transfer of CLC had less impressive anti-tumor effects, but the results indicate some potential to the approach to immunotherapy by production and transfer of large numbers of cytotoxic CLC. The in vivo radioisotopic assay has been used to assess the in vivo significance of in vitro detected suppressor cells for NK activity.

As a model of cell-mediated immunity against tumor associated antigens, detailed investigations have been performed with continued cultures of human T cells (CTC), maintained with T cell growth factor. The cytotoxicity mediated by CTC from patients with solid tumors has been examined. Observations indicate possible anti-tumor activity in these CTC populations. Attempts have recently begun to clone these effector cells by limiting dilution, in the presence of feeder layers and TCGF.

The role of macrophages in resistance to cancer has been a topic of major interest to this laboratory. Macrophages have been shown to direct cytotoxic effects against tumor cells, act as accessory cells for the generation of immune responses, and to act as suppressor cells.

Macrophages from several strains of mice were activated by various agents and tested for their cytolytic activity against a panel of tumor targets. In addition, several monosaccharides were tested for their ability to inhibit macrophage-mediated cytotoxicity and for comparison, their effects were also tested on NK activity. Data from both models indicate heterogeneity of recognition or lysis by macrophages. Investigators have probed the possibility that the functional and biochemical changes that occur during the activation of mouse macrophages were accompanied by modifications in their macromolecular synthesis. Experimental data support the hypothesis that inhibition of macromolecular synthesis, particularly an early inhibition of RNA synthesis, is required for activation of macrophages.

Studies of inhibition of tumor growth by human peripheral blood monocytes were continued in a variety of assay systems. Growth inhibitory activity (GIA), measured by effects on tritiated thymidine incorporation, was found to be increased after pretreatment of effector cells with phorbol ester or IF. A new microassay system for the human GIA, which allows drastic reduction in the number of cells required for its performance, has been validated in terms of reproducibility and statistical norms.

Human monocyte function was also assayed by measuring cytotoxicity against tumor cell lines by a variety of isotope release assays. Accessory function by human monocytes has been assessed by their ability to facilitate the production of TCGF by CTC. Two myelo-monocyte human cell lines were found that produce IL-1, and cooperate with T cells, inducing their TCGF production. They represent an easy approach towards the analysis of the requirements for cell surface structures and mediators that are involved in such cooperation.

The ability of different populations of mouse macrophages to suppress the production of macrophage activating factor (MAF) by stimulated T-lymphocytes was investigated. It was found that activated macrophages, infiltrating MSV-induced regressing tumors or recovered from the peritoneum of mice-injected with C. parvum, were able to actively suppress the production of MAF. The macrophages

suppressed the early events of lymphocyte activation leading to MAF production. It seems likely that there are different subpopulations of macrophages, which vary in their stage of activation or differentiation. Experiments are underway to develop monoclonal antibodies against cell surface antigens on mouse and human macrophages in an attempt to provide reagents that could identify subpopulation of macrophages. Similar attempts are in progress to produce monoclonal antibodies to subpopulations of human monocytes.

Work on the purification of a human lung-associated antigen (hLTAA) which is highly organ site restricted has been completed. A radioimmunoassay using ^{125}I-labeled antigen has given early indication of clearly discriminating lung cancer patients' sera from those of normal, healthy individuals. Recent examination of urines from patients with lung cancer has indicated the presence of material which is immunologically realted to LT-120 antigens. The antigen, was also found in the urine of patients with other malignancies (melanoma, multiple myeloma, mycosis fungoides).

A primary objective of LID is to develop and evaluate tests for their possible application to clinical problems of cancer patients. Therefore a laboratory has been set up for detailed immunologic monitoring of cancer patients. Procedures have been established to standardize and control the assays and to minimize various sources of technical variations in results. A major emphasis has been placed on the use of cryopreserved cells.

Much attention has been focused on the possible therapeutic efficacy of IF. IF has been shown to substantially boost NK activity. To determine the optimal doses and schedules of IF administration, the highly purified recombinant leukocyte IF A preparation of Hoffman-La Roche is being used to treat patients with advanced cancer. A fixed dose of IF, ranging from 1 to over 100 million units is being given for 28 days. These patients are being carefully monitored for the effects of the treatment in the range of immunologic parameters listed above.

The research program of the Laboratory of Immunobiology, directed by Dr. Hebert Rapp, continues to study immunologic mechanisms that impede tumor growth and spread in vivo and in vitro. The results of these studies have served as a basis for the design of clinical protocols to test the efficacy of immunotherapy for patients suffering from malignant melanoma or from head and neck cancer.

Studies of active specific immunotherapy have proceeded along two major paths: evaluating efficacy of active specific immunotherapy in guinea pigs with different stages of malignant disease and modifying vaccines in an attempt to improve immunotherapeutic potency. It was shown that immunization of guinea pigs with a mixture of irradiated syngeneic tumor cells plus mycobacterial cell walls in an oil water emulsion eradicated tumor cells remaining in lymph nodes after limited surgery for stage IIexperimental cancer and prevented the progression of the disease to stage III. Tumor implanted intravenously in the lungs of animals after limited surgery for stage 2 disease was also eliminated by immunization. This vaccination was not effective in guinea pigs with a large tumor burden. Here, successful vaccination required injection of a mixture containing irradiated tumor cells and heat killed whole mycobacterial cells.

Studies of the intralesional immunotherapy with natural and synthetic analogs of bacterial components have continued. It was shown that a single intralesional

injection of an emulsified mixture of trehalose 6,6' dimycolate and synthetic muramyl dipeptide could cause tumor regression and elimination of lymph node metastases in a guinea pig with an established dermal tumor and regional lymphoma node metastases. A lysozyme digest of a peptidoglycan obtained from a Gram positive bacerium, Brevibacterium divaricatum could substitute for muramyl dipeptide. Evidence has been obtained indicating that antigens coded for by endogenous murine leukemia virus may function as common tumor rejection antigens on chemically-induced murine fibrosarcomas.

Adoptive transfer of spleen cells from specifically immunized donors to non-immunized recipients was used to study tumor immunity in vivo to the syngeneic line-10 guinea pig hepatoma. Hepatoma cells cultured as monolayers on fibronectin coated surfaces served as targets for immune splenocytes in a tritium release cytotoxicity assay in vitro. These studies revealed that the immune cells mediating cytotoxicity in vitro were functionally distinct from those conveying adoptive protection in vivo. Adoptive tumor immunity appears to require no host response and the transferred cells are randomly distributed in the skin.

The effectiveness of mycobacterial components in the eradication of pulmonary deposits of intravenously injected syngeneic fibrosarcoma 1023 in C3H mice was studied. BCG cell walls (BCG,CW), trehalose 6,6'-dimycolate (TDM), or 6,6'-di-O-2-tetradecyl-3-hydroxyoctadecanoyl trehalose were administered in emulsified form, intradermaly or intravenously. The most effective therapy was TDM given by the intraperitoneal route.

A single intravenous dose of the carcinogen N-nitroso N-methyl urea was used to produce malignant breast tumors in female Buf/N rats. Treatment with surgery of immunotherapy using BCG CW resulted in the regression or arrest of the tumor in a significant proportion of rats.

The role of immunoglobulins (Igs) in activating the complement (C) sequence has been further elucidated. Previous work showed that sera of several mammalian species contain significant levels of naturally occurring anti-methotrexate (MTX) and anti-folinic acid (FA) IgM antibodies. Methods were developed to determine the number of haptens bound to the cell surface and the number of IgM molecules attached to the cells carrying different amounts of the hapten. It was found that the naturally occurring human IgM anti-MTX and anti-FA antibodies bound to cells in three different forms.

It was concluded that the binding of IgM to the cell by one antigen reactive site of the IgM molecule is sufficient to bind C1 but not sufficient to activate the lytic sequence. The mechanism of complement fixation differed for different classes of immunoglobulins.

Efforts continued to elucidate the mechanism whereby lectins initiate, augment or inhibit activation of C in the presence or absence of Igs. Succinylated and acetylated concanavalin A (Con A), but not the native lectin specifically activated the lytic C sequence on sheep cells in the absence of Igs. Other lectins with the same or different sugar specifically either augmented or inhibited hemolysis. Augmentation by Con A was consistent with the ability of the lectin on the cell surface to bind but not to activate C1. Thus cell bound Con A and IgG under conditions where IgG by itself could not activate the lytic-C sequence

appeared to yield a complex that behaved like an aggregate of IgG molecules. These observations suggested the hypothesis that the activation depended on the IgG in the complex. Characterization at the molecular level of the reaction between protein A of Staphylococcus aureus and Igs of various species and classes has also continued. The enhancement of the reaction between protein A and affinity purified rabbit IgG to human serum albumin correlated with immune aggregation but not with antigen induced allosteric changes in the Ig molecule.

These studies showed that, in addition to the immunoglobulin class, acquisition of a biological activity by Igs, depends on the size, molecular composition, distribution and density of the immobilized ligand. Whether the activated, potentially cytolytic C sequence can kill a cell depends, on the target cell itself. Pretreatment of guinea pig tumor cells with certain metabolic inhibitors, enzymes and X-irradiation increased, while pretreatment with certain hormones decreased their susceptibility to killing by C. These effects were in general ascribed to the synthesis and composition of cellular lipids and other physiological properties of the cell. Similar studies were performed using two human lyphoid cell lines, PY and Raji and the mouse mastocytoma cell line P815. These cells show variable susceptibility to C mediated attack during different parts of their growth curve. In contrast to the guinea pig cells, susceptibility or resistance so far did not correlate with lipid synthesis. Additional studies support the observation that physiological properties of cells determine their susceptibility to killing by C. Immunological interactions of host and tumor are another major area of interest for this Laboratory. Control of macrophage nonspecific effector function has been analyzed by several appraches. Macrophages from certain strains of mice fail to develop nonspecific tumoricidal activity after any of several in vivo or in vitro treatments over a wide range of experimental conditions. Deficits in tumoricidial activity by cells from BCG-infected C3H/HeJ or A/J mice have been shown to result from mechanistically different defects in macrophage-tumor cell interaction.

Regulation of nonspecific macrophage effector function is mediated by several different lymphokines. Despite the apparent homogeneity of lymphokine activity for induction of macrophage tumoricidal effects, at least two functionally different signals can be identified. Macrophages respond to one mediator (priming signal) to enter into a noncytotoxic but highly receptive state in which they can then respond to a second lymphokine stimulus (trigger signal) to develop tumoricidal activity. It is the trigger signal that is limiting during immune reactions. Lymphokine priming and trigger signals from the basis of a regulatory system that sets the threshold and determines the onset of macrophage effector function. Development of tumoricidal activity by macrophages treated in vitro with lymphokines follows a reproducible time course. Macrophage activation for tumoricidal activity correlate with alterations in cell lipid content. In vivo studies also indicate that macrophage activation for nonspecific tumoricidal activity is associated with alterations in cell lipid content.

In a quantitative study of chemotactic responses of blood monocytes from normal human subjects, it was found that only a fraction of the total monocytes migrated at optimal attractant concentration and incubation time. The size of this responsive subpopulation was between 25 and 40% of the total, depending on the donor, and was not affected by various alterations of in vitro conditions. The response appears to be a simple interaction of individual cells with chemoattractant, leading to directional movement. In contrast, similar analysis of

mouse resident peritoneal macrophages revealed that the size of the responsive population increased with time, and the percentage of input macrophages that migrated toward attractant increased with an increase in cell concentration. Thus, responsiveness of mouse macrophages appears to be affected by cell-cell interaction on the chemotaxis membrane surface, and at the higher cell concentrations there is extensive cell-cell contact.

Comparison of the characteristics of migrating and non-migrating leukocyte populations was made possible by the design of a separation chamber, a modification of the 48-well chemotaxis chamber. The purpose of the study was to compare chemoattractant binding to migrating and non-migrating populations of human monocytes.

The research effort of the Laboratory of Cell Biology, directed by Dr. Lloyd Law, has emphasized the following areas: histocompatibility antigens, tumor antigens, transfection with tumor DNA, immunoglobulin structure, genetics and diversity, and mammalian cellular genetics. A primary focus of the work of this laboratory has been the study of the biochemistry and structure of modecules. The chemical and biological properties of murine histocompatibility antigens (H-2) have been studied. The purpose has been to purify the molecules expressing these antigens, to determine their properties at each stage of purifications, and when they are reconstituted into artifical membranes composed of lipid. It is thought that by examining the behavior of these molecules in simple reconstituted membranes, an understanding can be gained of how these molecules interact with receptors on lymphoid cells in order to express their known biological effects on T cell mediated immune reactions.

Biochemical isolation of histocompatibility antigens, transformation related protein (p53), tumor antigens and epoxide hydrase has been carried out. The chemical structure of these proteins is being approached by micro methods and the relationship of the amino acid sequence to other known structures is being analyzed. Cloning of histocompatibility antigens, B_2 microglobulin and the p53 protein is being approached in order to study the molecular organization of these systems and permit a better understanding of the nature of the extensive polymorphism at the H-2 loci and the mechanism involved in the regulation and expression of different genes.

The major transplantation antigens (SLA) from inbred herds of minature swine are under study. Papain fragments of these antigens have been prepared and purified. Amino terminal sequences have been obtained for the heavy chains as well as the associated B-2 microglobulin. DNA probes have been used to characterize the genes coding for these antigens by Southern blot analysis and to isolate genomic clones containing transplantation sequence.

Major emphasis has been placed on the study of tumor antigens of the transplantation rejection type (TSTA), and of tumor antigens assayed by in vitro techniques and of the immune responses they evoke. Solubilization and methods of purification of both TSTA and H-2 are under investigation with the ultimate purpose of defining these membrane antigens after purification in physicochemical, biologic and molecular terms. The role of a B-tropic retrovirus as a vector in the transmission of genetic information of a specific tumor antigen is under study as well as the use of TSTA markers in the hope of defining more clearly the molecular events in transfection studies using DNA of chemically-induced sarcomas.

Much of the research effort has focused on the study of immunoglobulin structure, genetics, and diversity. Studies include: the karyotypic analysis of plasmacytomas, susceptibility to plasmacytoma induction, galactan binding myeloma proteins, VK-isotypes, and studies of organization and control of genetic material in immunoglobulin-secreting plasmacytomas.

It has been shown that the pathogenesis of peritoneal plasmacytoma development in BALB/c mice involves a genetically-determined component and a pathological tissue microenvironment that selects preplasmacytoma cells. Identification of genes controlling susceptibility is being approached by making BALB/c congenic strains. Mechanisms involved in the selective proliferation of pre-plasmacytoma cells on oil granuloma is being studied in vivo and in vitro. Amino acid sequence analysis of hybridomas with specificity for B(1.6) galactan is in progress to evaluate structure, function, and idiotype. Nucleic acid probes are being developed to characterize the gene structures coding for these proteins.

Studies in the field of mammalian cellular genetics are continuing. The purpose of this project is to analyze and develop new cell systems in culture. Fisher rat thyroid cells (FRTL) are being grown in low or no serum. These cells are the first and possibly the only example of a karyotypically normal, long term cell strain that has retained complex differentated functions. These cells are hormone dependent. They synthesize and secrete a very large protein product, thyroglobulin. They concentrate iodide 100-fold from the medium. They offer a unique opportunity to study secretion, ion uptake and cAMP response.

The effect of interferon in murine retroviruses is studied concentrating on pertinent factors which influence cell differentiation and malignant transformation, using techniques and approaches ranging from the microscopic to molecular level. Special emphasis is placed on understanding the mechanisms whereby whole virions are rendered non infectious. The study of the role of retroviruses in oncogenesis and tumor immunogenesis investigates the role of type C retroviruses as an etiologic agent and a vector of genetic information for neoplasma, and the immunologic responses of the host to tumor associated antigens. In this regard, work continues on characterization of newer isolates of xenotropic viruses a dual-tropic virus and amphotropic viruses by competitive radioimmunoassays. Investigations continue on the interferon and natural killer (NK) activity associated with transplantable and sponteneous reticulum cell neoplasms (RCN) of SJL/J mice. It has been demonstrated that tumor cells can trigger the host's own suppressor mechanism via macrophages to suppress the T cell-mediated cytotoxic response. It appears that tumor cells can utilize the host's own immune system to evade the host's immune surveillance.

The clinical research program of the Metabolism Branch, directed by Dr. Thomas Waldmann, has two major goals: The first is to define host factors that result in a high incidence of neoplasia. The second major goal of the Branch is to determine the physiological and biochemical effects that a tumor produces on the metabolim of the host.

A primary research effort has been to define the major events of cellular differentiation, cellular interaction and cellular biosynthesis involved in the specific circulating immune response. Emphasis has been placed on defining the defects of immunoglobulin genes, the patterns of rearrangement and deletion that regulate B cell differentiation, and on defining the factors in normal and abnormal states that control the production of antibodies and the synthesis of the immunoglobulin molecules.

The earliest events of maturation of stem cells into B cells involve rearrangements of the genes coding for the appropriate light and heavy immunogloblin chains. 32p labelled clones of human kappa (C_K) and lambda ($C\lambda$) constant region genes have been used as probes to study gene rearrangements in lymphocytes from chronic lymphocytic leukemia patients, Epstein-Barr virus transformed and spontaneous B-cell lines. Experimental evidence suggests an ordered hierarchy with kappa gene activation preceeding lambda. In contrast to B cells, human T cell leukemias almost always retained their immunoglubulin genes in the germ line configuration. To study the earliest events of immunoglobulin gene recombination eight human "non-T", "non-B" leukemic lymphocytes representing immature stages of lymphoid differentiation have been examined. Seven of these populations demonstrated immunoglobulin gene rearrangements indicating that most of them are already committed to B-cell development at the immunoglobulin gene level. Such populations of B-cell precursors, rich in recombinational errors of immunoglobulin gene joining, may indicate that the somatic assembly of immunoglobulin genes is remarkably prone to error.

The maturation of B cells into antibody producing plasma cells is carefully regulated both positively and negatively by distinct subpopulations of lymphoid cells. Suppressor T cells have been known to emerge from the thymus as inactive prosuppressor cells that require an interaction with another T cell in order to become a final effector of suppression. A series of techniques has been developed to study the terminal differentiation of B cells into immunoglobulin synthesizing and secreting cells, to assess the helper T cell function, and to detect both increased and decreased functional activities of suppressor effector T cells, their precursors and activators.

To approximate the in vivo situation, investigators have initiated studies of immunoglobulin class specific antigen-specific antibody production that do not require polyclonal B cell activators. A new culture and assay system was developed for the sensitization of human peripheral blood mononuclear cells with a T cell dependent antigen in the absence of nonspecific stimulatory agents with the subsequent generation of macroscopic hemolytic plaques. It was shown that the antibody produced by the plaque forming cells generated in this culture system is specific for the sensitizing antigen, and that the plaques created are not false plaques, and that the antigen specific response measured by this system is dependent on adherent cells and T lymphocytes. This system is simple, sensitive, and should serve as an effective tool for the analysis of cellular interactions involved in the generation of human antigen specific PFC, the genetic control of the human immune response, and the pathophysiology of altered immunoregulation in disease.

Alternative methods have been developed to measure specific antibody responses by human peripheral blood mononuclear cells in vivo and these methods have been used to study defects in the immune responsiveness of patients with various immunodeficiency states. Influenza viruses were used as antigens, and antibody production was measured by an Enzyme-Linked ImmunoSorbent Assay (ELISA). The production of antibody was shown to require the cooperative interaction of T-cells, B-cells and monocytes in culture. This requirement for T helper cells capable of promoting the maturation of B-cells into antibody secreting cells was further investigated using a series of hybridoma derived monoclonal antibodies directed at predominantly distinct subsets of human T-cells.

Work continues on the study of Epstein-Barr virus (EBV) as an activator of human B cells into immunoglobulin secreting cells. EBV infects cells of the immune system almost exclusively, specifically the B lymphocytes. Recent evidence suggest a potential relationship between EBV and rheumatoid arthritis (RA). Patients with RA have elevated serum antibody titers to certain EBV associated antigens. EBV rapidly induces B cell lines from the peripheral lymphocytes of RA patients and EBV infected B cells produce rheumotoid factor in culture. The immunoregulatory T cell function has been studied in 20 patients with RA to determine whether a defect in the function of such cells might be present in this disease. The EBV specific suppressor T cell which inhibits EBV induced B cell activation in normal immune subjects, was markedly deficient in these RA patients, even though they were immune to EBV as determined by the presence of antibodies to EBV in their serum. Thus, patients with RA exhibit a profound defect in immunoregulatory T cell function with is restricted to involve the response to EBV. This defect, which prevents EBV induced B cell activation, may be an important contributor to the immunologic basis of this disease.

The mechanisms and sites of action of suppressor T cells continue to be a major area of interest. In most of the suppressor cell assays of human B cell responses, the site of action of the suppressor cell is unknown. Four separate and distinct suppressor T cell systems have been defined and all four of these suppressor T cells were shown to act directly on the B cell. Using the in vitro biosynthesis procedures with polyclonal activation, patients with disordered immunoglobulin synthesis have been identified. These include disorders in the intrinsic B cell activity, helper T cell function, the interacting T cells involved in immune suppression and in monocyte function.

Leukemic T cells with retained functions have been analyzed using monoclonal hybridoma antisera that define T cell subsets. These studies support previous conclusions that Sezary cells are relatively mature T cells that are dedicated to helper interactions with B cells. Studies on three patients with T cell leukemias with retained suppressor activity have been continued in order to address the question of whether suppressor cell activity is generated by a single population of T cells acting alone or whether suppressor cell generation requires interaction between different populations of T cells neither of which has the capacity to affect suppression alone. Experiments support the hypothesis that an interaction between at least two different T cell subsets is required for the generation of so-called suppressor-effector T cells in man. Additional experiments indicate that there is cooperative interaction between neoplastic and normal T cells in the generation of immunoregulatory effector T cells.

A modification of the immunoglobulin biosynthesis procedure has been developed to assess the ability of macrophages to act as one of the helper cells required for B cell maturation and to detect activation of monocytes into cells that inhibit immunoglobulin synthesis. It appears that normal macrophages can be activated to inhibit B cell maturation and immunoglobulin synthesis. Patients with multiple myeloma have a disorder of the monocyte suppressor system with excessive numbers of activated suppressor monocytes that lead to the reduced polyclonal immunoglobulin synthesis observed with these patients. A similar monocyte suppressor disorder associated with a monocyte produced suppressor factor has been demonstrated in mice bearing transplantable myeloma tumors.

In order to study the mechanisms of determinant-specific Ir gene control of the antibody response to myoglobin in vitro, a modified Mishell-Dutton culture system has been developed in which secreted antibody specific for myoglobin can be measured in the culture supernatants by a solid phase immunoassay. This in vitro antibody response was found to be macrophage and helper T-cell dependent. In order to dissect the cell interactions involved in mediating this Ir gene control, cell populations from different strains were mixed in combinations which would not produce an allogeneic effect. Although one could not exclude the possibility that residual F_1 macrophages are presenting antigen, it was impossible to demonstrate an Ir genetic restriction on macrophage function in this in vitro secondary antibody response. Since an Ir restriction was observed for B cells plus macrophages, it is possible that in the in vitro secondary antibody response to myoblobin, Ir gene function is involved in T cell-B interactions but not in macrophage-lymphocyte interactions.

Other investigators have directed their efforts towards understanding the cellular mechanisms regulating mucosal immune responses in general and IgA immunoglobulin responses in particular. Prior studies indicate that the regulation of the IgG response was independent of the IgC and IgM responses. To determine the cellular basis of this observation studies were initiated on the properties of cloned regulatory T cells maintained in continuous culture with interleukin 2 (IL-2), a T cell growth factor. Two Peyer's patch T cell lines, two spleen T cell lines and one colonic Peyer's patch T cell line have now been obtained. Clonal frequency analysis determined by limiting dilution and Poisson distribution indi cates that each clone has in fact originated from a single cell. Analysis of re-cloned cells supported this conclusion. Each of these cloned cell lines was characterized and tested for its capability to serve as helper cells for inducing production of IgA and other immunoblobulins, and each was capable of helping B cells produce IgA. Thus, IgA-Fc receptors are not necessary to help IgA synthesis. Preliminary studies indicate that these cloned T cells can initiate suppression of Ig synthesis of T cells on Sephadex-anti-$F(ab')^2$ columns.

Primary biliary cirrhosis (PBC), a progressive inflammatory disease of the liver, is not susceptible to treatment with corticosteroids. Studies have shown that when cell cultures containing syngeneic mixtures of purified B cells and T cells obtained from PBC patients were set up, the T cells failed to manifest the suppressor cell activity on PWM-stimulated Ig synthesis that is normally manifest at high T cell/B cell ratios. These studies indicate that the autologous mixed leukocyte reaction (MLR) results, at least in part, from surface antigens expressed on cells (B cells) after cell activation. In addition, they show that the main functional consequence of the autologous MLR is the activation of a suppressor T cell population. This suggests that through the autologous MLR, cell activation leads to feedback suppressor induction and that in the autologous MLR one has a polyclonal equivalent to feedback suppressor circuits previously described in antigen specific systems.

The mechanism of action of cytotoxic mononuclear cells and their contribution to host defense has been a topic of prime concern. Special emphasis has continued to focus on studies of antigen-specific immune responses by human peripheral blood leukocytes in vitro. Influenza viruses have been employed as model antigens for the investigation of cell mediated cytotoxic responses by thymus derived lymphocytes, T-cells and humoral antibody responses by B-cells whose maturation into immunoglobulin secreting plasma cells is both positively and negatively regulated by T-cells.

Humans with immunodeficiency diseases have recurrent infections, and a greatly increased incidence of neoplasia. These patients' lymphocytes have been studied for their capacity to generate influenze virus immune-cytotoxic T-lymphocyte response (CTL). Experimental evidence suggests that in certain immunodeficient patients who lack humoral immune responsiveness to viruses in vivo, CTL effector responses may be retained and contribute to host responsiveness. The inability to generate CTL in other immunodeficient patients may contribute to the increased incidence of infections and neoplasic observed in association with these diseases.

In other studies of cell mediated cytotoxicity, investigators have been working on the characterization of the molecular requirements for the expression of synergistic cytotoxicity. This model which measures the ability of human serum factors to activate human monocytes and lymphocytes to kill erythrocyte targets is now substantially characterized.

Studies continue on the characterization of the pharmacology of spontaneous monocyte cytotoxicity, a model which measures cell mediated lysis of target cells in the absence of serum factors. These studies have been expanded to clinical studies of cancer patients and early results demonstrate excellent correlation with in vivo and in vitro data. These studies suggest a mechanism resulting in activation of cytotoxic monocytes by agents which are normally thought of as toxic. Such activation may play an important in vivo role. Anti-inflammatory drugs used in the treatment of rheumatoid arthritis can enhance monocyte function as well.

Additional effort has been focused on understanding porphyrin metabolism. Succinylacetone (4,6-dioxoheptanoic acid), an irreversible inhibitor of ALA dehydrase, (the second enzyme of the heme biosynthetic pathway) has been studied for its effects on tumor cells in vitro and in vivo. This compound can markedly inhibit the growth of the Walker 256 carcinosarcoma and Novikoff hepatoma, however, it has had only slight activity in vivo against leukemis L1210 in mice. Other studies indicated that succinglacentone has a profound immunosuppressive effect. The antiproliferative effect of succinyl-acetone results from inhibition of heme biosynthesis in some cells (murine erythroleukemis cells), but operates by a heme independent mechanism in other cells (Walker 256 and Novikoff).

The Laboratory of Pathology provides the diagnostic services in Pathological Anatomy for the Clinical Center of the NIH and has research programs in various areas of experimental pathology.

The Surgical Pathology and Postmortem Section is responsible for the surgical pathology and autopsy diagnostic services. 5,666 surgical specimens or biopsies were accessioned in the past year. 141 autopsies were accessioned. These specimens involved the preparation of 53,408 slides, including 36,023 H&E stained sections, 6,238 special stains and 605 frozen sections. Clinico-pathological studies in pulmonary vasculitis, breast cancer, pancreatic carcinoma, esophageal carcinoma, recurrent hyperparathyroidism and soft tissue sarcoma, are in progress.

The Cytopathology Section provides diagnostic services in cytology (both exfoliative and fine needle aspiration) and medical genetics. The chromosomal

analysis includes conventional and special banding techniques for the examination of individual chromosome pairs. During the year, approximately 3600 cytology specimens were accessioned. Collaborative research projects include: CSF evaluation of leukemic patients to assess the therapeutic effects reflecting the degree of relapse or remission; chemotherapy effects on the GU tract as seen in urine specimens; chemotherapeutic and radiation effects on the exfoliative cells (sputum, fluids, vaginal smears, etc.) and maturation index in precocious babies.

The Ultrastructural Pathology Section provides diagnostic electron microscopy services. This year 254 cases were accessioned; over 175 were processed and diagnosed. Collaborative efforts include a study of the effect of IgE cross linking which indicated IgE was slowly shed but not internalized unless cross-linked by dimethyl suberimidate. This phenomenon was shown to be independent of histamine release. A rigorously purified monomeric IgE-ferritin conjugate has been used as a label of cellular IgE disposition. Initial studies reveal a previously unappareciated non-uniformity of cell surface labelling as well as apparent internalization of some label. The IgE-ferritin conjugate has also been used to label membrane vesicles derived from basophils, in a multifaceted study of the IgE-Fc receptor complex. Results suggest that the Fc_ε receptor is not a transmembrane protein.

Work on human tumor tissue and human tumor cells in vitro continues. Significant progress in the detection and qualitative analysis of tumor cell synthesized procollagen and collagen has been made. A variety of tumors and their cultured progeny are being studied, and preliminary results support a strong association between tissue of origin and collagen type synthesis - i.e., osteosarcoma, of bone origin synthesizes almost exclusively type I collagen.

The Biochemical Pathology Section is carrying on research on the immunochemistry of complex carbohydrates. Current approaches include determination of carbohydrate structures of glycoproteins and analysis of oligosaccharide mixtures by gas chromatography/mass spectrometry, development of hybridoma antibodies against oligosaccharide haptens, and studies on the origin, metabolism, and excretion of a urinary oligosaccharide derived from glycogen.

Interactions between polyoma (Py)-transformed salivary epithelium (PTSE) and normal fetal mouse dermis have been studied to investigate the epithelial-mesenchymal interactions during and after Py transformation. It was found that PTSE induced melanin synthesis when placed in contact with normal melanoblasts in fetal mouse dermis. In a corollary experiment, normal salivary epithelium interacting with normal dermis was found to form hair follicles complete with sebaceous glands and well-developed hair shafts.

Isozyme studies of Py-induced tumors of several epithelial types showed profiles for 30 enzyme systems to be constant for each organ of origin, but different from organ to organ except for the salivary and mammary glands, which had identical profiles. In addition, polyoma tumors derived from salivary epithelium transformed by Py in vitro differed in their isozyme signatures from salivary epithelial tumors induced in vivo. These results indicate that the phenotype of polyoma-transformed cells is largely dependent on the phenotype of the target cell at time of transformation.

X cell lesions (papillomas and pseudobranchial tumors) found in fish have been investigated to determine whether they are actually of host fish-cell origin. Experimental results from a variety of techniques indicate that X cells are parasitic rather than neoplastic in nature.

Studies in the Viral Oncology and Molecular Pathology Section focus primarily on human viruses, and the mechanisms by which these viruses interact with cells susceptible to lytic productive infection and with cells which can undergo malignant transformation.

Work continues in the biologic and virologic characterization of breast and prostate cancer cells. In studies involving retroviruses in mouse mammary tumor cells, it was found that cells which initially synthesized only a type B retrovirus (MMTV) with passage continue to produce increasing amounts of type C retrovirus. This was accompanied by a marked increased in the synthesis of type C viral RNA but not by a decrease in the MMTV RNA. Mouse interferon inhibited the production of both viruses equally but had no effect on the intracellular concentrations of the two viral RNAs.

Work continues on the human and bovine papillomaviruses. Collaborative studies have shown that the BPV-1 and BPV-2 DNAs which have been cloned, are capable of transforming susceptible rodent cells and that a subgenomic transforming segment of the BPV-1 genome is capable of inducing this transformation. Other investigators have participated in collaborative studies designed to exploit the extrachromosomal nature of the BPV genome in transformed mouse cells, and to demonstrate the utility of this DNA as a cloning vector in eukaryotic cells.

Research continues on the immunologic, cytochemical, biochemical, and functional aspects of human malignant lymphoma. Over 125 fresh biopsy specimens have been analyzed during the past year from in house lymphoma patients. A study on the correlation of immunologic phenotype with conventional morphology, has shown that immunologic phenotype cannot be predicted by morphologic criteria alone. The clinical importance of immunologic phenotype in patients with diffuse aggressive non-Hodgkin's lymphoma is being analyzed.

Another major research effort has led to the development of a system by which 15 to 38-fold increases in the levels of translatable fibronogen mRNA could be induced in the livers of Sprague-Dawley rats by inducing defibrination with Malayan pit viper venom. cDNA clones for the α, β, and γ chains of fibrinogen have been obtained. They have shown that the genes are not independent but are coordinately regulated. This unique approach could be used to obtain recombinant plasmids for other families of coordinated regulated genes.

The Pathological Technology Section has provided the histological services and staining procedures for scientists in DCBD and other divisions of NCI. During FY 81 they processed more than 18,000 pieces of animal tissue, cut and stained more than 47,000 H&E slides and prepared over 6,000 special stained slides. They also prepared over 13,000 unstained for immunoperoxidase studies and other special techniques.

Investigations continue on the role of interaction between stromal and hematopoietic cells in the micro-environmental support of hematopoiesis. Marrow-derived adherent cells (MDAC) have been shown to provide a microenvironment which

supports hematopoiesis in vitro. Experiments have shown that these MDAC are stromal in origin, and that these stromal derived monolayers support hematopoiesis in continuous bone marrow culture. Previous studies showed that MDAC consist largely of collagen-producing cells. Further biochemical studies have been undertaken to quantitate and to characterize collagen synthesis by stromal cells in intact marrow and in MDAC culture. Projects currently in progress include studies of the role of fibronectin in the stromal hematopoietic interaction, studies of proteoglycan synthesis by bone marrow stromal cells, and studies of the synthesis of connective tissue proteins by marrow stromal cells in Steel anemic (Sl/Sl-d) mice.

The Image Processing Section has extended its biological and biomedical related computer science research to center around the following major areas: computer-aided 2 dimensional polyacrylamide SDS gels, nucleic acid morphology, durable fiber (asbestos, fiber glass, etc.) effects on living cells, and biologically oriented image processing research. A 3-year plan for the replacement and upgrading of the RTPPs with provision for color graphics was developed and its first stage implemented by procurement of a COMTAL dual user display.

A variety of support software and hardware projects, necessary for the biologic research programs have been completed. Prominent among these was the final derivation and implementation of a pointwise shade correction algorithm that treats otherwise uncorrectable inhomogeneities in illumination and/or detection fields.

A biologically oriented image processary utility package for the System-20 has been specified and developed.

The Dermatology Branch, directed by Dr. Stephen Katz, conducts both clinical and basic research studying the etiology, diagnosis and treatment of inflammatory and malignant dieseases involving the skin and the host's response to these diseases. The basic research involves biochemical as well as biological studies of skin. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center

The Dermatology Branch is continuing to evaluate the safety and effectiveness of new oral and topical agents particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne. Oral 13-cisretinoic acid is effective in the treatment of these diseases. An oral synthetic aromatic derivative of retinoic acid has been similarly tested and found to be more effective and less toxic than 13-cis-retinoic acid in the treatment of the disorders of keratinization, while a topical synthetic aromatic retinoid was found to be ineffective A high initialdosage of 13-cis-retinoic acid followed by low maintenance was found to be an comfortably effective schedule and less toxic than the continuous high-dosage schedules in the treatment of cystic acne.

The morphologic and biochemical effects of Vitamin A and its analogs on skin have been evaluated. Topical all-trans retinoic acid, but not systemic 13-cis-retinoic acid, increased gap junction density and decreased desmosome density in treated basal cell carcinomas, thus indicating that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms.

Patients with xeroderma pigmentosum (XP) are particularly susceptible to the carcinogenic action of UV-radiation and develope multiple malignancies on sun-exposed areas of skin. Most XP patients have a marked impairment in the rate and/or efficiency of DNA repair. Understanding the relationship between DNA repair deficiency and skin tumor development in XP patients would elucidate the role of DNA repair in preventing those cancers which may be due to certain chemical and physical carcinogens. Studies of the DNA repair defects may also provide an understanding of the relationship of DNA repair processes and abnormal aging. Recent studies have been conducted with cells from patients with neurological lesions similar to those seen in some XP patients and in patients with ataxia telangiectasia. The studies are designed to elucidate the pathogenesis of these disorders as well as to develop presymptomatic diagnostic tests. The biological effectiveness of DNA repair was assessed primarily by in vitro assays of cell survival after treatment of the cells with DNA damaging agents. The trypan blue exclusion test has been used to determine the number of lymphoblasts surviving after irradiation with x-rays.

Hypersensitivity to the lethal effects of N-methyl-N-nitrosoguanidine (MNNG), a mutagenic and carcinogenic DNA-damaging chemical has been found in fibroblasts from patients with the following disorders: Huntington disease, infantile spinal muscular atrophy, spinocerebellar ataxia familial dysautonomia, tuberous sclerosis, muscular dystrophy and retinitis pigmentosa. This hypersensitivity may provide the basis for a prenatal diagnostic test for these diseases in tissues from high risk fetuses. In addition, cytogenetic studies were initiated to determine whether DNA-damaging agents induce abnormal numbers of chromosome abnormalities in cells from patients with primary neuronal degeneration. The development of such a cytogenetic test would make it possible to detect hypersensitivity to DNA-damaging agents within a few days, as opposed to the currently employed colony-forming test which requires two to three weeks.

Studies of the immunopathology of skin diseases have focused on two areas. The first is cell mediated immunity and the role of epidermal Langerhans cells in the induction and expression of cell mediated immune responses. It was demonstrated, using a simultaneous rosetting and immunofluorescence technique and X irradiated bone marrow reconstituted mice, that epidermal Langerhans cells are derived from precursor cells in the bone marrow. These Langerhans cells have been shown to be critical for antigen presentation in vitro as well as in vivo.

The effects of UV light on the ability of Langerhans cells to induce sensitization have been explored. Although UV has no effect on surface marker characteristics of Langerhans cells, UV light irradiation of epidermal cells and their subsequent haptenation and injection into syngeneic mice abrogates their ability to sensitize. Indeed, these UV irradiated Langerhans cells induce specific suppressor T cells which are responsible for the hyporesponsiveness which results. Additional collaborative studies have shown that epidermal cells, devoid of Langerhans cells, and epidermal cell cultures produce factors with Interleukin 1-like activity. This factor has been termed ETAF (epidermal cell derived thymocyte activating factor). The biological functions and biochemical characteristics are currently under study.

Studies continue on the abnormal proteins produced in malignant melanoma. These studies are aimed at elucidating the mechanism of formation of these atypical proteins, as well as their importance to the immunology of melanoma and possible

immunotherapy potential. Melanosomal proteins from melanoma tissues vary in structure from those of normal tissue. Several of the proteins seem to be missing completely from these granules, which are also structurally distinguishable from normal granules by electron microscopy. Many of the proteins in melanoma melanosomes are not found in normal melanin granules. In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules revealed several differences. Peptide mapping demonstrated that amino acid sequences are detected in 3 or more regions of the abnormal protein. Other proteins in these tissues seem to differ in a similar manner. It has been found that tumor-specific proteins similar to these can be found in the serologic fluids of melanoma patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro.

SUMMARY

Annual Report of the Laboratory of Biochemistry, National Cancer Institute
October 1, 1980 through September 30, 1981

I. INTRODUCTION

This summary and the detailed reports that follow document a busy and productive year in the Laboratory of Biochemistry. Many highly interesting new findings have been made, and an atmosphere of lively discussion and cooperation prevails. In part this is because of the nature of our own research, but it also reflects the air of excitement generated by the spectacular progress in biochemistry and molecular biology world-wide.

During the year, Dr. Edward Kuff was appointed Deputy Laboratory Chief. The administrative duties involved in managing a Laboratory of this size are substantial, and yet, the most important tasks for the Laboratory Chief are scientific. The new arrangement will allow the Laboratory Chief to share some duties, leaving more time for attention to the scientific program.

Another organizational change that occurred this year was the appointment of Dr. Martin Rosenberg as Chief of the Section on Cellular Regulation. His tenure in this position assures a high level of activity in this important area. Also, Dr. Rosenberg has been, and will continue to be, an important factor in our ability to attract young investigators interested in the regulation of gene expression both in prokaryotes and eukaryotes. In this connection, we are also happy to report that Dr. Dean Hamer joined the Laboratory this year as a Senior Staff Fellow and is establishing his own independent research program within the Section on Cellular Regulation.

The constant participation of members of the Laboratory in meetings and seminars both at NIH and at other institutions here and abroad has kept us all in touch with the latest research news. And this was bolstered by the many excellent investigators who have visited us and presented seminars to the Laboratory.

The Laboratory has a large and first-rate group of Staff Fellows, Visiting Fellows, Visiting Scientists, Guest Workers, Experts, and summer students, who contribute good ideas, hard work, critical conversations, and fellowship to our community. Summertime has been particularly lively because of the weekly seminar program for summer students, held jointly with the Laboratory of Molecular Biology.

To be candid, it is necessary to mention some worrisome developments. We are sorry that this year's Summer Student Program was curtailed. Many young people were disappointed at the lost opportunity to learn first-hand about research, and we were disappointed to have so few young and inquiring minds to stimulate our thinking and help with our experiments. We are even more concerned that the continuing restrictions on hiring...the freeze...seriously hamper our efforts to ensure that the laboratories will be able to welcome young, temporary investigators, who are so essential to our productivity and scientific modernity. Because of the prevailing customs in biochemistry, recruitment of the best qualified individuals proceeds a year or two in advance.

Under the present circumstances we are already losing the most attractive candidates.

II. BIOCHEMISTRY OF GENE EXPRESSION SECTION (Dr. E. B. Thompson, Chief)

This has been an unusually successful year. Dr. Thompson and his colleagues achieved several goals which provide the basis for interesting future experiments.

The human glucocorticoid receptors have been purified sufficiently to use for the induction of antisera in rabbits. The plan now is to characterize these sera fully and to use them for the assay of receptors in both steroid-sensitive and -resistant cells. First, cultured cells will be used and later cells from patients who have steroid-treatable diseases. The antiserum (or its IgG) will also be bound to columns to purify the receptors further. With enough receptor of >90% purity the group will seek to: 1) make monoclonal antibodies and 2) obtain partial amino acid sequence data.

Dexamethasone mesylate, synthesized by a collaborator, S. S. Simons, was shown to be a covalent affinity ligand for glucocorticoid receptors. A nondissociable label for these proteins should assist greatly in their purification, in analysis of the structure of their ligand-binding sites, and in comparative analysis (as by 2-D gel electrophoresis) of receptors between species, tissues, and especially between sensitive and resistant cells. Dexamethasone mesylate is also a long-acting glucocorticoid antagonist; it is hoped that it will prove useful in physiologic studies of receptor synthesis and turnover.

The rat growth hormone (GH) and prolactin (Prl) genes isolated in genomic clones are now being sequenced. About ~ 675 bases have been sequenced at the 5' end of the GH gene, and a number of Prl restriction fragments have been cloned preparatory to sequencing. A cell-free transcription system will be set up and parts of those genes will be used as templates. The rat and mouse GH gene content of L, GH_3, and L x GH_3 cell hybrids have been analyzed with eight restriction endonucleases. Hybrids, both GH-expressing and nonexpressing, contain GH genes from both parents in virtually identical configurations. Little or no GH mRNA is synthesized in the nonexpressing hybrids. The intention is to microinject GH and Prl-specific and -nonspecific DNA's and L cell proteins into GH_3, L and hybrid cells to test the theory that L cell proteins are specifically shutting off GH and Prl production in the hybrids.

Human leukemic (CEM) cell tumors have been established in nude mice and preliminary data indicate that an unorthodox steroid is a more effective anti-leukemic agent than are traditional glucocorticoids. Unselected clones of CEM cells are sensitive to glucocorticoids and show wide variability with respect to the time of continuous exposure to steroid which is required before the onset of growth inhibition. A clone of CEM cells not lysed by but containing functional receptors for glucocorticoids has been found. It is hoped that this clone will provide information about the "post-receptor" steps in steroid action.

A computer-assisted image-analysis method for screening clones of DNA-bearing bacteria is being developed and tested on the library of cDNA's from steroid-treated rat liver.

III. BIOSYNTHESIS SECTION (Dr. E. L. Kuff, Chief)

The Section has three independent research groups.

A. (Dr. Beverly Peterkofsky and coworkers). This group has continued its studies on the regulation of collagen synthesis in normal and transformed cells. They earlier found that sarcoma virus-transformed mouse fibroblasts have a marked increase in the synthesis of type III collagen relative to the usual type I. Productive infection and transformation of BALB/c 3T3 cells with a temperature-sensitive Kirsten sarcoma virus have now shown that this virus-specific phenotypic alteration persists at the non-permissive temperature. This unusual behavior, which is being actively investigated, resembles a terminal differentiation and suggests either that the src protein is not involved in the change or that it initiates the change but the reaction is essentially irreversible. Studies have also continued on the role of ascorbic acid in collagen metabolism in guinea pigs. In addition, a microsomal reductant which can substitute for ascorbate as a cofactor for prolyl hydroxylase has been partially purified from L-929 cells: it is a hydrophobic protein which can be separated into high and low molecular weight forms by gel filtration.

B. (Dr. Samuel Wilson and coworkers). The group is studying the enzymes and ancillary factors involved in DNA replication. They have examined the mechanism of E. coli DNA polymerase I large fragment using simplified reaction systems containing either $(dT)_{800}$ or $(dA)_{800}$ as template. This DNA polymerase was capable of highly processive DNA synthesis, incorporating several hundred dNMP residues per cycle of binding to the template, incorporation, and termination. They found that termination at any given product chainlength was markedly increased by the presence of higher concentrations of the template·primer complex. This and results of steady-state kinetic and enzyme/template binding studies suggested that the polymerase has more than one functional site for interaction with polynucleotides. Tryptic peptide mapping of putative DNA polymerase α subunits indicated that there is sequence homology between higher M_r (110,000) and lower M_r (50,000-65,000) polypeptides found in homogeneous preparations of the enzyme from calf thymus. A number of hybridoma clonal lines have been generated that appear to be producing monoclonal antibody to calf thymus α-polymerase.

C. (Drs. Edward Kuff and Kira Lueders, and coworkers). This group has pursued studies of integrated intracisternal A-particle (IAP) genes in the mouse. They have shown that these genes, which are reiterated to the extent of 1000-2000 copies per genome in all strains of Mus musculus thus far examined, have structural aspects similar to the proviral DNA of type B and C retroviruses, i.e., the genes are colinear with the IAP 35S RNA and have terminal repeat segments (TRS's) several hundred base pairs in length. TRS's from several isolated IAP genes have been subcloned and their nucleotide sequences are being examined for possible regulatory signals. The genetic relationship between IAP's and an infectious extracellular virus (M432) from an Asian mouse species has been determined by heteroduplex analysis of the cloned genes: M432 appears to be a recombinant that has incorporated a major block of IAP sequences into the central (putative polymerase) region of the viral genome. Additional studies have shown that sequences homologous to the IAP genes are endogenous to all mouse species tested and to several other rodent genera; they are particularly well conserved in the Syrian hamster. A systematic survey has revealed several instances of IAP genes in proximity to other known mouse

genes. Recently a translocated pseudo α-globin gene in BALB/c mice has been found to be bracketed by two IAP genes.

IV. CELLULAR REGULATION SECTION (Dr. Martin Rosenberg, Chief)

The work of the Section is carried out by two independent groups.

A. (Martin Rosenberg and coworkers). Recombinant DNA techniques have been used to develop plasmid, phage, and bacterial vector systems which allow the isolation, characterization, and comparison of prokaryotic transcriptional regulatory signals. This system is being used to study the efficiencies with which various promoter and terminator signals function. In addition, the system allows selection of mutants in a variety of sites and the precise correlation of the structural alterations with their functional effects. Thus the system has been used to monitor the strengths of various promoters and the ability to regulate transcription from these signals when present on a high copy number plasmid. This information has led to the development of another vector system which has the potential of (over)producing essentially any protein within the bacterial cell. A strong promoter, which can be regulated effectively in high copy number, has been fused adjacent to efficient prokaryotic ribosome binding sites. Coding information for a variety of prokaryotic and eukaryotic gene products is being fused into these systems. The ability of these systems to produce these proteins will be assessed.

The above system has been used successfully to overproduce a 97 amino acid polypeptide encoded by phage λ , which serves as a positive activator of transcription by RNA polymerase. Large amounts of the protein have been obtained and a variety of physical and biochemical studies on this molecule have been initiated. Interactions of this protein with itself, with DNA, and with RNA polymerase are being studied.

Previous work demonstrated that under the appropriate conditions prokaryotic genes could be expressed efficiently in eukaryotic cell-free systems. Recently Dr. Rosenberg and his colleagues have demonstrated efficient expression of the E. coli galactokinase gene directly within mammalian cells. High levels of the bacterial enzyme galactokinase (galK) are produced in a variety of mammalian cells transfected with an SV40-plasmid recombinant vector carrying the E. coli galK gene. On this vector, the galK coding sequence was inserted downstream from the SV40 early promoter so that the translation start codon for galK became the first AUG on the transcript. To ensure proper transcript maturation, SV40 regulatory sequences for RNA splicing and polyadenylation are incorporated beyond the galK coding region. Cells transfected with this vector produce large amounts of a new galK activity which is similar, by starch gel electrophoresis, to that found in E. coli.

Our ability to obtain expression of an easily assayable gene product from a structurally defined fusion vector now permits studies of: (1) functional complementation by the bacterial enzyme of primary cells isolated from human patients with galK-deficiency galactosemia or other galK-deficient mammalian cells; (2) transcriptional regulation in mammalian cells by fusing various eukaryotic promoters to the galK gene; and (3) translational regulation in mammalian cells by selectively altering the primary structure of the 5' nontranslated portion of the transcript.

B. (Dean Hamer and coworkers). The general objective of this group is to understand the regulated expression of animal cell genes. Towards this end they have constructed simian virus 40 (SV40) recombinants carrying a variety of chromosomal eukaryotic genes. These recombinants, together with a complementing helper virus, have been introduced into cultured monkey cells where they replicate to levels of 100,000 copies per cell. Such experiments allow examination of regulatory sequences, such as promoters and splicing sites, and also production of substantial quantitites of useful gene products, such as human growth hormone and hepatitis B surface antigen, in cultured cells.

V. DEVELOPMENTAL BIOCHEMISTRY SECTION (Dr. Igor B. Dawid, Chief)

This section is composed of two independent groups.

A. (Igor Dawid and coworkers). This group has continued its study of the structure and expression of the genes for ribosomal RNA (rRNA) in Drosophila melanogaster. They have determined the nucleotide sequences in several regions of these rRNA genes. These include the region in which transcription terminates, and the boundaries between rRNA regions and insertions of type 1 and type 2. The termination region is of interest with respect to the fact that it does not show the string of T residues commonly found at this position. The boundaries of two type 1 insertions show target site duplications typical of transposable elements. In contrast, type 2 insertion boundaries do not show such duplications. These data suggest that interrupted rRNA genes arose by the insertion of transposable elements into rDNA, leading to the inactivation of these genes.

A study of genome organization in D. melanogaster has been carried out in collaboration with M. L. Pardue of MIT, M. Gans of the CNRS at Gif-sur-Yvette, France, and V. Pirrotta at the EMBO Lab in Heidelberg, Germany. The organization of repeated DNA elements interspersed with insertion-like sequences has been studied. These interspersed elements were shown to be homologous to sequences located on different chromosomal sites in different stocks of D. melanogaster, suggesting that the sequences are transposable in the genome. One such transposable element, named 101F, has been analyzed further. We have shown that it is a member of a divergent family of sequences, does not carry long terminal repeats, and that its insertion leads to a duplication of 13 base pairs at the target site. These properties define a new class of transposable sequences in Drosophila.

The structure and expression of the vitellogenin genes in Xenopus laevis has been studied in collaboration with G. U. Ryffel and R. Weber of the University of Bern, and W. Wahli of the University of Lausanne, Switzerland. In X. laevis, vitellogenin which is synthesized in the liver under control of estrogen is encoded in a small family of genes. Two of the vitellogenin genes together with long stretches of their flanking regions have been isolated from a X. gene library. The structural organization of these two genes has been determined by electron microscopy. In both genes, the mRNA coding sequence is interrupted 33 times by sequences (introns) not present in mature vitellogenin mRNA. The distribution of repeated DNA sequences in and around the two vitellogenin A genes has been determined. Many of the introns contain sequences that are repeated elsewhere in the genome. It is suggested that intron evolution may involve the insertion and deletion of mobile repeated DNA elements.

Gene expression during the development of X. laevis has been studied in collaboration with M. B. Dworkin and J.W.B. Hershey of the University of California in Davis. This study employs a set of cloned cDNAs which have been prepared from poly(A)$^+$RNA molecules of two embryonic stages of X. laevis. In a library of 200 cloned sequences that code for abundant RNAs in two embryo stages, transcripts of some structural and some mitochondrial genes have been identified. A significant proportion of cDNAs in this library contains sequences that are moderately repeated in the genome. To analyze stage-specific RNA species, a DNA library is being constructed containing particular RNAs which are present in gastrula embryos but absent in oocytes. To analyze the developmental expression of defined genes, cDNA clones for two calcium binding proteins, calmodulin and parvalbumin are being generated. These experiments are expected to give results that will aid in an understanding of gene activity during development and generate a set of developmentally regulated genes that provide useful material for further study.

B. (Bruce Paterson and coworkers). This group has studied gene expression during muscle development in the chicken. The proteins encoded in the most abundant mRNA class expressed in embryonic chick muscle have been identified and include the following: Myosin heavy chains (M-HC), myosin light chains (M-LC), α and β actin, tropomyosin, vimentin, desmin, glyceraldehyde phosphate 3' dehydrogenase (GAP), muscle and brain creatine phosphokinase (CPK). Certain isozymic forms of these proteins are expressed only in differentiated muscle, e.g., myosin heavy and light chains, α actin, and muscle CPK, whereas all cells express the other forms of these proteins. In addition, the muscle specific set of proteins appears to be coordinately regulated in that all these genes are switched on synchronously during differentiation. Using defined, cloned ds-cDNA probes for these genes, genomic sequences for, α and β actin, vimentin, myosin heavy chain and GAP have been isolated. The organization, structure, and regulation of the set of differentiation specific and the constitutive genes is being compared and there is a focus on α and β actin and vimentin.

VI. MACROMOLECULAR INTERACTIONS SECTION (Dr. Claude B. Klee, Chief)

The section is concerned with the mechanism of regulation of cellular processes in eukaryotes by the Ca^{2+} receptor protein, calmodulin. Previous studies have shown that under physiological conditions of ionic environment the sequential binding of Ca^{2+} to the four Ca^{2+} sites of calmodulin is accompanied by stepwise conformational transitions. These enable the protein to translate quantitative changes in Ca^{2+} levels into qualitatively different cellular responses because different target proteins recognize different Ca^{2+}·calmodulin conformers. The five different Ca^{2+}·calmodulin conformers are being characterized further by limited proteolysis and fluorescent probes to facilitate study of the mechansm of the Ca^{2+}-dependent interactions of calmodulin with its several target proteins (M. Oldewurtel and M. Epstein).

A unique feature of calmodulin is its ability to interact with a large variety of different enzymes and proteins. Using methods which have been developed in this laboratory for the study of the regulation of cyclic nucleotide phosphodiesterase by calmodulin, in collaboration with Drs. Cohen, Adelstein and Neer, it was shown that other enzymes such as phosphorylase kinase, myosin kinase and adenylate cyclase also interact with calmodulin. In each case the interaction is between the catalytic subunit of the enzyme and calmodulin The Ca^{2+} dependence of interaction of calmodulin with its acceptor proteins

and of the subsequent activation of the enzymatic activities is different. As a consequence, calmodulin can regulate the extent of responses to a Ca^{2+} signal as well as the temporal sequence of the responses. Studies on structural relationships between the different calmodulin-dependent enzymes are now being pursued to characterize the molecular mechanism of these specific interactions. Calmodulin-dependent phosphodiesterases from different tissues and different mammalian species are structurally extremely similar but different from other known molecular forms of the enzyme (Krinks and Takemoto).

A third aspect of the mechanism of action of calmodulin is the coupling with cAMP-dependent cellular responses. In addition to the roles of Ca^{2+} and cAMP as regulators of each other's intracellular concentrations, each of these second messengers affects the responses of its targets to the other regulator as was shown in the case of myosin kinase and phosphorylase kinase. An interaction between calmodulin and the regulatory subunit of cAMP-dependent protein kinase in brain preparations has also now been demonstrated. This interaction results in an increased affinity of the protein kinase's catalytic subunit for its regulatory subunit. The multiple links observed between the two messenger systems provide a frame work upon which the intricacies of cellular responses to external stimuli can be built.

VII. NUCLEIC ACID ENZYMOLOGY SECTION (Dr. Maxine Singer, Chief)

There are two groups in this section.

A. (Maxine Singer and colleagues). This group is studying the relation between the genomes of simian virus 40 (SV40) and the African Green Monkey (AGM). During a productive permissive infection of AGM cells by SV40 the virus coopts the cell's resources for its own reproduction. The virus is not passive in the process: after the host's RNA polymerase ribosomes, etc, synthesize the viral encoded T-antigen, this protein in turn stimulates the synthesis of cell-encoded enzymes required for DNA synthesis. Cellular DNA synthesis occurs, followed by the T-antigen dependent construction of new viral genomes. These observations suggest a significant relation between the structures of the SV40 and AGM genomes. During the past year we investigated this relation using recombinant DNA techniques to isolate from the AGM genome, DNA segments related to SV40. For this purpose we prepared a library of the AGM genome in λ bacteriophage.

The library yielded 3 different AGM DNA segments that hybridized, under stringent conditions, to the region around the SV40 origin of replication. Subcloning and primary nucleotide sequence analysis established that the 3 SV40-like regions each comprise a few hundred base pairs in which several different and short sequences are homologous to viral sequences. The order of the sequences is scrambled in the AGM segments compared to the virus. The homologies include segments known to be involved in viral DNA replication, T-antigen binding, and the control of both early and late viral transcription. The ability of the AGM segments to function as origins of replication and as "promoter"-like elements is being investigated. Preliminary experiments with one of the AGM segments have been carried out in collaboration with Paul Berg's laboratory using SV40-based vectors constructed there. The SV40 control regions of the vectors are replaced by the monkey sequence and AGM cells are transfected with the resulting molecules. The available data are encouraging but tentative and

suggest that while the AGM segment is not likely to be a replication origin, it may well be a component of a complex promotor.

A second type of AGM DNA segment which has been isolated from the library is a sequence known to occur in defective recombinant variants of SV40. From one variant, a segment homologous to an infrequent AGM sequence was purified by molecular cloning and used to select a homologous region from the genomic library. Characterization of this sequence proceeds.

Another interest of this group is the structural organization of the highly repeated DNA of the African Green Monkey (AGM). One highly repeated DNA of AGM is largely contained within a cryptic satellite called α-component. The overall structure of α-component consists of long tandem repeats of a segment 172 base pairs in length. About 5×10^6 copies of the segment occur in the genome, largely confined to centromeric regions. We determined a nucleotide sequence corresponding to the most frequent nucleotide at each of the 172 positions in the monomer unit. Evidence was presented indicating that nonrandom variants of the average sequence occur. More recently, primary nucleotide sequence analysis of 8 cloned copies of the monomer sequence, selected at random, demonstrated that the "repeat" units of α-component include a large number of sequences, each differing in one or a few residues from the average. Work in other laboratories suggested that for α-component and similar highly repeated DNAs in other species, related variants may exist together in genomic domains perhaps within specific chromosomes.

The α-component sequences in single monkey chromosomes isolated by construction of somatic cell hybrids between AGM cells and mutant rodent cells is now being studied. Thus far one cloned cell line carrying a single monkey chromosome (with a gene for thymidine kinase) has been obtained. Restriction endonuclease digests of DNA from these cells have been probed with cloned α-component and compared with similar digests of total AGM DNA. The results show that the isolated chromosome contains a family of α-component sequences markedly different from the entire set present in AGM. A library of the hybrid cell line DNA in lambda bacteriophage has been constructed and phage containing sequences that hybridize with α-component are being analyzed.

In another approach to studying α-component organization a library of total AGM DNA in lambda phage was constructed in such a way as to select against the most abundant type of α-component organization, namely, long tandem repeats with no EcoRI site. Analysis of 16 clones has confirmed that special groups of variant α-component sequences are in hand. Of the 16 phage at least 11 contain junctions between α-component and other genomic sequences. Two subfamilies of these AGM segments can be defined on the basis of common junction sequences. These junction sequences will be purified by molecular cloning in order to study their abundance in the genome and to investigate whether they occur only as junctions with the satellite or in other positions. In this way reconstruction of a chromosome around the centromeric region can begin.

A new line of research involves an attempt to establish an *in vitro* system for RNA-splicing (removal of intervening sequences). The system is designed to address two of the problems encountered in previous attempts. First, abundant purified substrate is being prepared *in vitro* using *E. coli* RNA polymerase and a vector containing SV40 early DNA under the control of a powerful promoter. Second, extracts are being prepared from F-9 murine teratocarcinoma cells before

and after the cells are induced to differentiate by retinoic acid. Earlier work suggested that one block to expression of T-antigen in undifferentiated F-9 cells was at the level of RNA processing. The block was overcome upon differentiation. Use of the paired extracts may provide useful controls and aid in understanding the nature of the block in the stem cells.

B. (Richard Kramer and coworkers). Dr. Richard Kramer and his colleagues have used recombinant DNA technology to explore the organization and expression of the yeast genome. The yeast Saccharomyces cerevisiae was chosen as a model system for this project because extensive genetic studies have been carried out with yeast and because yeast has a relatively small genome. While yeast is a relatively simple eukaryotic organism, it has many molecular biological properties similar to those of higher organisms and not found in prokaryotes. Thus, yeast provides a relatively simple system for studying eukaryotic gene organization and expression at the molecular level.

Two yeast genes, each present in multiple copies in the yeast genome, were examined. One codes for a repressible acid phosphatase (APase) and the other for the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In both cases, several of the multiple copies were isolated.

The APase gene copies were isolated by differential plaque filter hybridization screening of a phage "library" for genes induced by growth in low-phosphate medium. The structures of the APase genes were determined by restriction enzyme analysis, DNA sequencing and heteroduplex analysis. The mRNA map positions were determined by R-loop analysis, S1 nuclease mapping and reverse transcriptase extension of a small DNA primer. One of the APase genes has been inserted into a yeast vector, and deletion mutations have been constructed to define the sequences required for regulation and expression of the APase gene.

The yeast GAPDH gene copies were isolated by plaque filter hybridization to the yeast library with a chicken GAPDH gene clone as a probe. This was possible because of the strong homology of the GAPDH enzymes of many diverse organisms. Structural analysis and RNA mapping were carried out for these genes by the same techniques used for APase.

VIII. PROTEIN CHEMISTRY SECTION (E. A. Peterson, Chief)

A. O. Wesley McBride and coworkers: In previous work, Dr. McBride and his colleagues transferred mammalian hprt and tk genes to eukaryotic cells by DNA-mediated gene transfer. These transformed cell lines are being used to clone the tk gene. For this purpose, DNA has been isolated from mouse cells that were serially transformed with human tk by DNA transfer. It was sheared to 15-20 Kbp fragments without loss of tk transforming activity, then size fractionated by velocity sedimentation, tailed with poly(dA), and annealed with EcoRI-cleaved, poly(dT)-tailed, Charon 9 vector arms. The recombinant DNA will be incubated with a λ phage packaging extract and used to transfect E. coli. The resulting library will be screened directly by transfer to nitrocellulose filters and hybridization with a human [^{32}P]-labeled repetitive DNA probe. Plaques containing human DNA inserts will be isolated and purified from the library consisting predominantly of mouse recombinants. A plaque containing the intact human tk gene will be identified by transformation of LMTK$^-$ cells with plaque-purified recombinant phage DNA preparations under

selective conditions. A variety of control studies have been performed, including filter hybridization of human repetitive DNA probes with mouse and human recombinant DNA libraries. It was shown that most human DNA fragments of 20 Kbp length contain at least one hybridizable sequence of human middle repetitive DNA, and that these sequences can be demonstrated under conditions in which there is no detectable hybridization with mouse DNA sequences. The human hprt gene will be cloned by nearly identical procedures.

Somatic cell hybridization has been used to prepare a panel of 25 independent human/rodent hybrid cell lines segregating human chromosomes. The specific human chromosomes present in each line have been determined by isozyme analysis, and assignments have been confirmed in some cases by karyological analysis. In collaboration with Dr. David Swan, DNA was isolated from each line, and gel blots were prepared after digestion with restriction endonucleases, followed by agarose gel electrophoresis. These blots have been used to map human immunoglobulin genes and MSV src genes to specific human chromosomes in collaboration with Drs. Philip Leder and Stuart Aaronson.

B. (Michael Mage and colleagues). His development, last year, of a preparative nonlytic separation of $Lyt2^+$ and $Lyt2^-$ T cells has enabled Dr. Mage to examine directly the question of help for the generation of cytotoxic effector cells (CTL). When highly purified $Lyt2^+$ responder spleen cells were put in primary mixed lymphocyte culture with semiallogeneic B cells as stimulators, no CTL were generated, despite the presence of their precursors in the $Lyt2^+$ population. However, when $Lyt2^-$ cells were added, CTL were generated to the same extent as with an unfractionated responder cell population.

In collaboration with Dr. John Farrar of the Laboratory of Microbiology and Immunology, NIDR, Dr. Mage has found that the lymphokine "IL2" can substitute for $Lyt2^-$ cells in providing help to splenic CTL precursors, resulting in the generation of CTL from highly purified $Lyt2^+$ responder spleen cells in primary mixed lymphocyte culture.

C. (Warren Evans and colleagues). Using the response of guinea pig bone marrow granulocyte precursors to inflammatory stimuli as a model for studying the nature of feedback regulators of granulocyte differentiation, Dr. Evans and his colleagues have shown previously that 12-hr inflammatory (S-12) serum has a pronounced stimulatory effect on the mitotic activity and on the glycosylation of granule components of bone-marrow granulocyte precursors in primary cell cultures. The results of chromatography of S-12 serum on Con A-Sepharose columns suggest that the mitotic- and the glycosylation-stimulating activities (MSA and GSA) are associated with separate, nondialyzable serum components.

In collaboration with Dr. Howard Terebello of the Hematology/Oncology Section at Walter Reed Hospital, information gained from the guinea pig model is being used to develop an assay for detecting factors in human serum that regulate the differentiation of human bone marrow granuloctyes. As in the guinea pig model, dialyzed serum from patients with recent infections stimulated glycosylation of subcellular components in human bone marrow granulocytes in primary cultures. This stimulating effect diminished as infection was controlled.

The transplantable granuloctyic leukemia (now referred to as GL-13 leukemia) previously developed in strain 13 guinea pigs is now in its 80th

transplant generation. In one transplant line the leukemia has recently undergone a significant change in its biological properties. As the white blood cell count increases above 30,000 to 50,000 cell per cmm, the leukemic population changes dramatically from a mixture of granuloctyes in which the more differentiated forms predominate to one in which a "blast crisis" occurs. This should make the GL-13 leukemia a valuable model for studies of the blast crisis in human leukemia, since there is at present no other animal leukemia in which this phenomenon occurs.

Cytogenetic studies of the GL-13 leukemia show a chromosome defect in these cells, a terminal deletion in one of the long arms of a single chromosome. In this characteristic, as well as in others reported previously, the GL-13 leukemia resembles the Philadelphia chromosome-positive (Ph^{1+}) type of human chronic myelocytic leukemia.

D. (E.A. Peterson and coworkers). Last year's report described a separation of the high mobility group nuclear proteins, HMG-1 and HMG-2, from each other and from other proteins of a 0.35M NaCl extract of calf thymus nuclei by displacement chromatography on DEAE-Sephacel with carboxymethyldextrans (CMD's) as displacers. It was observed in that work that the addition of CM-D to the sample kept the chromatin proteins in solution at low salt concentrations, making it possible to chromatograph them by elution as well as by displacement. A useful separation has been achieved in this way, but the low mobility group proteins were not as well separated from HMG-2 as in displacement chromatograms.

A small protein spot that appears in 2-dimensional gel electrophoretic patterns obtained with serum from psoriatic patients by the Anderson procedure has provided a convenient and interesting model for testing the use of narrow-range CM-D for the isolation of marker proteins from serum and the use of 2-dimensional gel electrophoresis for tracking them in the course of their purification. A high level of enrichment was achieved in the first pass through a DEAE-Sephacel column at pH 7.1, using narrow-range CM-D and applying a volume of serum equal to 43% of the column volume. (In a later experiment this was increased to 86% without seriously affecting the separation.) The fractions containing the desired protein were pooled and chromatographed at pH 8.4 on another column of the same type and size, yielding the desired protein in almost pure form, as shown by 2-dimensional gel electrophoresis. It was readily separated from the CM-D accompanying it by adsorption on a small column of CM-cellulose at pH 5, followed by sharp elution with ammonium acetate. Its identity as the psoriasis-related protein was established by mixing a sample with a trace of the original serum to provide a background for reference and subjecting the mixture to 2-dimensional electrophoresis.

The isolated protein still contained a substantial amount of Gc globulin, identified by its neighboring position in the gel pattern. Antibody prepared with the product after separation from Gc globulin by isoelectric focusing developed a single band against both psoriatic and normal serum in an Ouchterlony diffusion assay and immunoelectrophoresis gave the same result. Displacement chromatography of a presumably normal serum yielded highly enriched fractions that clearly contained psoriasis-related protein.

IX. OFFICE OF THE CHIEF

Dr. Cecil Fox is detailed to the Armed Forces Institute of Pathology, where he carries out his research. The goals of this program are to use biological characteristics of tumor cells as a means for improving grading of human cancer through use of more objective parameters. The object of grading tumors is to estimate the potential of the tumor for growth, invasion, and metastasis, which are important factors to clinicians in planning therapy. Tumor cell attachment to new substrates is being compared to normal cell attachment using reflection contrast microscopy. A major characteristic of cancer cells, release from density dependent growth control, is also being studied as is the role of lamellar cytoplasm in tumor cell growth. The distribution of enzyme sites on the membrane surface of human oncogenic fibroblasts has been compared to the distribution in nononcogenic cells. Some new projects characterize human cancer cell populations in archival tissues and with needle aspiration cytology. A study of endometrial epithelium and its growth dynamics in relation to endometrial hyperplasia is now underway, and a separate project on the dynamics of cellular response to cytostatic agents in head and neck cancer is in the process of activation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00333-18 LB

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Basis for Defective Differentiation in Granulocytic Leukemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	W.H. Evans	Research Chemist	LB NCI
Others	E.A. Peterson	Research Chemist Chief, Protein Chemistry Section	LB NCI
	S. Wilson	Biologist	LB NCI
	M. Mage	Immunochemist	LB NCI
	D. Moore	Biologist	LB NCI

COOPERATING UNITS (if any)

Hematology, Oncology Section, Walter Reed Army Medical Center

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main thrust of this work is to develop biochemical methods for the early diagnosis of granulocytic leukemia and methods for inducing leukemic cells to develop some or all of their functional properties as a means of partially or completely restoring host defense mechanisms in leukemia patients. Work is first aimed at establishing which of the many biochemical steps involved in normal granulocyte differentiation are controlled by humoral regulators. The results will be compared with those obtained from similar studies on leukemic cells at corresponding stages of maturity in order to determine the nature and potential reversibility of the arrested differentiation steps. Biochemical analyses are carried out on mature and immature granulocytes isolated from blood and bone marrow and the effects of external cell regulators on granulocyte differentiation, as measured by changes in the synthesis of specific cellular components, are studied in a defined culture system previously developed in this laboratory.

PHS-6040
(Rev. 2-81)

Methods Employed: Leukocytes are isolated from bone marrow, blood and inflammatory exudates by a variety of methods. Subcellular fractions are prepared by differential centrifugation. Proteins extracted from leukocytes and serum proteins are fractionated by column chromatography and gel electrophoresis. Glycolipids are separated by thin layer chromatography and analyzed by gas-liquid chromatography and mass spectrometry. Immunological reagents, including fluorescent antibodies, are prepared against granulocyte proteins and used to follow their formation during granulocyte maturation. Stationary tissue culture methods are employed to study the synthesis of specific proteins from radioactive precursors. The distribution of radioactive proteins in electrophoretic gels is determined by slicing the gels in a gel fractionator and counting the slices in a liquid scintillation counter. Densitometric patterns of gels stained for glycoproteins are obtained by scanning the gels in a spectrophotometer equipped with a gel scanner accessory.

Major Findings:

A. Detection and Purification of Regulators of Granulocyte Differentiation in Guinea Pig Bone Marrow

We have shown previously that dialyzed, 12-hour inflammatory (S-12) serum has a pronounced stimulatory effect on the mitotic activity and on the glycosylation of granule components of bone marrow granulocyte precursors in cell cultures. Studies of the effect of S-12 serum on ^{3}H-thymidine incorporation into these cells indicate that DNA synthesis is also stimulated by inflammatory serum. The mitotic stimulatory activity (MSA) in S-12 serum has a low affinity for Con A-Sepharose columns, whereas the major portion of the glycosylation stimulatory activity (GSA) binds tightly to such columns. These findings suggest that MSA and GSA are associated with separate non-dialyzable serum components. GSA is not extracted from S-12 serum by lipid solvents and appears to be a glycoprotein since it is readily dissociated from Con A-Sepharose by methylmannoside. GSA also binds tightly to DEAE-cellulose columns at pH 7.0, suggesting that it is associated with a serum component that has a strong negative charge.

B. Development of an Assay for Regulators of Differentiation of Human Bone Marrow Granulocytes

In collaboration with Dr. Howard Terebelo of Walter Reed Hospital, we are using information gained from the guinea pig model system to design an assay for detecting factors in human serum that regulate the differentiation of human bone marrow granulocytes. In the human studies, bone marrow samples are obtained from the iliac crest of normal volunteers. Cell suspensions consisting of 93% granulocytes with 21% immature forms are prepared from bone marrow aspirates by hypotonic saline lysis and differential centrifugation. The cell culture conditions used to assay for differentiation regulators are essentially the same as those we are using in the guinea pig model system. As a potential source of differentiation regulators, dialyzed serum from patients with recent infections is employed. Using ^{14}C-glucosamine incorporation into granulocytes as a preliminary indicator of glycoprotein synthesis, we find that, as predicted on the

basis of the studies with the guinea pig model, serum from patients with infections markedly increases this measurement in human granulocytes in culture. Furthermore, this stimulatory effect diminishes as the infection is controlled. The results suggest that a humoral mediator is involved in the regulation of granulocyte glycoprotein synthesis.

C. Characterization of Leukemic Granulocytes

Cytogenetic studies of the leukemic cells from the GL-13 guinea pig leukemia developed in this laboratory indicate the presence of a chromosome defect in these cells. This chromosome alteration consists of a terminal deletion in one of the long arms of a single chromosome. In this characteristic, as well as in others reported previously, the GL-13 leukemia resembles the Philadelphia chromosome-positive (Ph^{1+}) type of human chronic myelocytic leukemia. No translocation of the deleted portion of the defective chromosome and no other chromosome abnormalities were detected in the GL-13 leukemic cells.

Significance to Cancer Research: (Objective 2, Approach 3). Granulocytic leukocytes are produced in the bone marrow by a complex, multistage, process of cell differentiation whereby proliferating, nonphagocytic, precursor cells are converted to nonproliferating phagocytic cells that play a crucial role in the body's defense against microbial invasion. Granulocytic leukemias can be thought of as diseases in which this program for differentiation is arrested at various stages, resulting in the overproliferation of immature cells lacking, in varying degrees, the capacity to carry out their normal function in the body's defense against infections. At present, the mechanisms involved in the initiation and modulation of the various steps in the differentiation program are poorly understood. Our research is aimed at clarifying the molecular processes that control the appearance of specific subcellular components during granulocyte differentiation. Such information should be useful in developing therapeutic approaches for reversing the arrested differentiation of leukemic cells.

Proposed Course of Research: The rate of differentiation of granulocytic leukocytes in the bone marrow is regulated by a feedback control mechanism which responds to the rate of destruction of these cells in the peripheral tissues of the body. Feedback information could be of a positive (stimulator) or negative (inhibitor) type transmitted by humoral and/or cellular factors, but at present the nature of these factors is unknown. We are using the response of precursors of guinea pig bone marrow granulocytes to inflammatory stimuli as a model for studying the nature of the feedback factors involved in the regulation of granulocyte differentiation. The humoral hypothesis for regulation will be examined by studying the effect of normal and inflammatory sera on granule-specific protein synthesis by granulocyte precursors in vitro. This approach is analogous to the use of hemoglobin as a marker for erythrocyte differentiation in assays for erythropoietin. Unlike erythrocytes, which contain only one major specific protein, granulocytes contain many potential marker proteins in their granules. Looking at this problem from a broader perspective, it is conceivable that the system we are

developing for the study of the coordinated regulation of macromolecular synthesis associated with secondary granule formation in myelocytes could serve as a useful model for studies of coordinate gene regulation in mammalian cells, just as the study of hemoglobin formation in erythrocyte differentiation has provided much information about the regulation of single genes. Our research plans are as follows:

(1) To further increase the specificity of our biochemical assay for regulators of granulocyte differentiation by employing highly enriched preparations of myelocyte precursors to study the initiation of secondary granule protein synthesis in cell culture.
(2) To purify secondary granule glycoproteins so that antisera against these proteins can be prepared for use in specific immunochemical assays for these markers of granulocyte differentiation.
(3) To isolate, purify and test in vivo any regulators of granulocyte differentiation detected by our assay.
(4) To apply the information obtained with guinea pigs to studies of the regulation of granulocyte production in humans.
(5) To search for defects in the granulocyte regulatory system in guinea pig and human leukemias.

Publications: None

Appendix: NIH Contract No. 1-60-25423

Funding: General NCI Contract for FCRC - No individual project funding breakdown available.

Man Years Purchased: 1

Major Findings

The transplantable granulocytic leukemia in strain 13 guinea pigs (now referred to as GL-13 leukemia) developed under this contract is now in its 80th transplant generation and has been successfully stored in the frozen state for up to 12 months without loss of biological activity. In one of our transplant lines, this leukemia has recently undergone a significant change in its biological properties. We are finding that as the number of leukemic cells in the blood increases above 30,000-50,000 cells per cmm, the cell population changes from a mixture of granulocytes in which the more differentiated forms predominate to one in which a "blast crisis" occurs and immature granulocytes (blasts and promyelocytes) predominate. This change, which takes place in the acute stage of the disease, is similar to the blast crisis that occurs in human chronic myelocytic leukemia. This new property of the guinea pig leukemia should make it a valuable model for studies of the blast crisis in human leukemia since at present there is no other animal leukemia in which this phenomenon occurs.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 00366-11 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biosynthesis and Assembly of Intracellular Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. L. Kuff	Chief, Biosynthesis Section	LB NCI
	K. K. Lueders	Chemist	LB NCI
OTHER:	L. Smith	Staff Fellow	LB NCI
	W. Kastern	Staff Fellow	LB NCI
	R. Callahan	Microbiologist	LVC NCI
	P. Leder	Chief, Laboratory of Molecular Genetics	LMG NICHD
	A. Leder	Biochemist	LMG NICHD

COOPERATING UNITS (if any)

Laboratory of Viral Carcinogenesis, NCI
Laboratory of Molecular Genetics, NICHD

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying murine intracisternal A-particles as a model for the evolution and expression of integrated reiterated viral genes. Multiple individual A-particle genes have been isolated from mouse DNA using recombinant DNA techniques. These genes, which number 1000-2000 copies per cell, make up a family of homologous but non-identical provirus-like elements which are highly conserved in various strains and tissues of Mus musculus. In the past year we have shown that sequences homologous to mouse IAP genes are present in other rodent genera and are particularly conserved in the Syrian hamster. We have extended our study of the genetic relationship between IAP's and an infectious extracellular virus derived from an Asian mouse species. The long terminal repeat segments of an IAP gene has been fully sequenced. We have begun to study the spatial relationship of IAP sequences to other mouse genes. A nuclear antigen has been detected in IAP negative cells using an antiserum raised against purified IAP structural proteins.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To study the mechanisms for coordinately regulating the synthesis of individual macromolecules and their assembly into complex intracellular components (organelles and viruses); specifically, to study the regulation of endogenous viral gene expression in relationship to normal development and neoplastic transformation.

Methods Employed: Culture of animal and bacterial cells; recombination and cloning of specific eukaryotic and viral DNA sequences in plasmids, lambda phage and retrovirus vectors; analysis of DNA components by restriction endonuclease cleavage, electrophoresis, and blot hybridization; chromosomal localization of specific DNA sequences by in situ hybridization; electron microscopy of DNA heteroduplexes and DNA:RNA hybrid molecules; nucleotide sequencing by procedure of Maxim and Gilbert; chromatographic and electrophoretic analysis of cellular and viral proteins; radio-immunoassay and immunoprecipitation of specific antigens; immunofluorescent staining.

Major Findings: Previous studies in this project have established that intracisternal A-particle (IAP) genes make up an extensively reiterated family of retrovirus-like elements in the cellular DNA of the Mus musculus. The basic genetic unit is 7.3 Kb long, colinear with 35S A-particle RNA, and contains terminal repeat sequences (TRS's) of about 350 base pairs. Genes containing deletions have also been observed. Recent findings are as follows:

(1) Comparative Studies. (a) Sequences homologous to the IAP genes of the mouse have been identified in the DNA of rat, gerbil, and hamster cells. These sequences are strikingly conserved in the case of the Syrian hamster, and the data suggest that the genome of this species contains multiple copies of a 6-7 Kb counterpart of the mouse IAP genetic unit. This is the first evidence that information related to the murine IAP is widespread in other rodent genera. (b) Earlier we showed that IAP sequences are fully represented in the DNA of two Asian mouse species, Mus cervicolor and Mus caroli, although in copy numbers of about 25 per haploid genome rather than 500-1000 as in M. musculus. We also showed that these endogenous IAP sequences have participated in the formation of an infectious extracellular virus (M432) isolated from M. cervicolor cells. Heteroduplex and restriction enzyme mapping of cloned IAP and M432 genes have defined the regions contributed by the IAP and those which are specific for the M432 virus (studies with Dr. Callahan, NCI). Sequences of the latter type (which include the M432 TRS's) are found only in M. cervicolor and one closely related species, whereas IAP-related sequences are endogenous to all mouse species thus far examined.

(2) Structural Studies. Both the 5' and 3' TRS's of a cloned IAP gene have been fully sequenced. The two 340 base pair TRS's differ from one another in 27 scattered positions, and lack several features (such as inverted repeats and short duplications of host flanking sequences) that are characteristic of freshly integrated retroviral proviruses. The data suggest that this particular randomly chosen gene copy was not generated by reverse transcriptase activity in the recent past. The LTR's contain nucleotide sequences that could be construed as signals for initiation and termination of transcription; however, direct

functional evidence for this is lacking. TRS's from two other IAP's are currently being sequenced.

(3) Spatial Relationships of IAP's to Other Mouse Genes. IAP genes are associated with most, if not all, of the mouse chromosomes (see previous Annual Reports). Since integrated proviruses can have effects on nearby genes, we have been testing for the proximity of IAP sequences to other known genetic elements. In collaboration with Drs. P. Leder and A. Leder (NICHD), we have found that a previously recognized pseudo-α-globin gene (αψ3) in BALB/c mice is closely bracketed by a pair of IAP genes. This spatial relationship is particularly interesting because some type of retroviral intervention has previously been suggested to account for the fact that αψ3 lacks the intervening sequences characteristic of functional α-globin genes and has been translocated to a different chromosome.

(4) IAP-Related Cellular Antigens. Rabbit antisera raised against purified IAP structural proteins have detectd a nuclear antigen in IAP-negative mouse cells. This antigen is observed by immunofluorescent staining in interphase but not mitotic cells. It reappears very quickly in the reconstituted daughter nuclei. The antigen appears to be reduced in the nuclei of cells that contain a large number of IAP's in their cytoplasm.

Proposed Course of Research: (1) We will continue to examine the spatial relationships between IAP sequences and other known genes, and to investigate the possible role of IAP's in the modification and translocation of other genes. The long terminal repeat segments of several more IAP genes will be subcloned, sequenced and tested for their capacity to promote transcription in experimental systems. The presence of active promoters in the TRS's would favor the possibility of downstream effects on nearby genes. (2) The comparative study of IAP-related sequences in other species will be continued. (3) Data will be sought to test the hypothesis that IAP's evolved from genes coding for normal cellular components, that such genes still coexist with their retroviral counterparts in the mouse genome, and that interactions between the normal and IAP-coded protein products can have physiological consequences for the cell.

Publications:

Kuff, E.L., Smith, L.A., and Lueders, K.K.: Intracisternal A-particle genes in Mus musculus: a conserved family of retrovirus-like elements. Mol. Cell. Biol. 1: 216-227, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00375-19 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Homogeneity and Structure of Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. A. Peterson	Chief, Protein Chemistry Section	LB NCI
OTHER:	A. R. Torres	Expert	LB NCI
	M. G. Mage	Immunochemist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Methods for the fractionation and analysis of proteins are developed and applied to the purification of specific proteins for the study of their function and structure. Displacement chromatography is being developed for the fractionation of macromolecules and particles of biological interest, employing polyanions differing in number of charges per molecule as displacers. Current efforts are directed toward the fractionation of nonhistone proteins of calf thymus nuclei and marker proteins in human serum. The procedure is particularly advantageous when large amounts of source material must be used to obtain sufficient amounts of a minor component.

PHS-6040
(Rev. 2-81)

Project Description

Objectives

To develop methods for the separation and analysis of proteins that will overcome present limitations and to apply these methods to the purification of specific proteins for the study of their functions.

Methods Employed

Protein fractions separated by ion-exchange displacement chromatography were evaluated by gel electrophoresis, using both denaturing and nondenaturing conditions. Carboxymethyldextrans (CM-Ds) having a variety of degrees of substitution were prepared by reaction of alkaline dextran (nominally 10,000 M.W.) with chloroacetic acid.

Major Findings

The pellet volume assay described in last year's report has proved to be more reliable than titration or the $A^{220}/\Delta n$ value for characterizing carboxymethyldextrans (CM-Ds) because it is insensitive to the contaminations that affect the latter measurements. The CM-Ds can be examined in the presence of proteins, as in chromatographic fractions, or in "pure" preparations.

The procedure described in last year's report for the fractionation of CM-D preparations has proven to be so effective as to permit the application of solutions containing a very wide range of affinities. This greatly decreases the total time and effort required for the preparation of the full range of narrow-range fractions.

Last year's report described a separation of the high mobility group nuclear proteins, HMG-1 and HMG-2, from each other and from other proteins of a 0.35 M NaCl extract of calf thymus nuclei by displacement chromatography on DEAE-Sephacel. Subsequent attempts to remove the CM-D accompanying the HMG fractions by adsorption of the protein on a column of CM-cellulose at pH 5, a procedure that has proved effective with other proteins, were unsuccessful because the HMG proteins were not adsorbed. Success has been achieved, however, by precipitating the HMG with 80% ammonium sulfate or by "salting it out" onto Phenyl Sepharose.

The addition of CM-D to the sample kept the chromatin proteins in solution at low salt concentrations and thus made it possible to chromatograph them by elution with NaCl. Although a useful separation was achieved in this way, the low mobility group proteins (LMG) were not as well separated from HMG-2 as in displacement chromatograms.

A small protein spot in 2-dimensional gel electrophoresis patterns obtained with serum from psoriatic patients by the Anderson procedure has provided a convenient and interesting model for testing the use of narrow-range CM-D for the isolation of marker proteins from serum and the use of 2-dimensional gel

electrophoresis for tracking them in the course of their purification. In a collaboration with Dr. Gerald Krueger of the University of Utah, who provided the sera, this protein was found in the sera of about half of the psoriatic patients studied and in that of 8 out of 9 patients who gave a positive Koebner response, i.e., developed psoriatic lesions in previously unaffected skin when that area was traumatized. The protein was detected at very low levels in some presumably normal sera.

A high level of enrichment was achieved by displacement chromatography with narrow-range CM-D on DEAE-Sephacel at pH 7.1, applying a volume of serum equal to 43% of the column volume. (In a later experiment this was increased to 86% without seriously affecting the separation.) The fractions containing the desired protein were identified by 2-dimentional gel electrophoresis, then pooled and applied to another DEAE-Sephacel column of the same size at pH 8.4, using narrow-range CM-D of somewhat higher affinity in anticipation of the higher affinity imposed on the protein by the increase in pH. After this second pass, the desired protein was obtained in almost pure form, as shown by 2-dimensional gel electrophoresis. It was readily separated from the CM-D accompanying it by adsorption on a small column of CM-cellulose at pH 5, followed by sharp elution with ammonium acetate. Its identity as the psoriasis-related protein was established by mixing a sample with a trace of the original serum to provide a background for reference and subjecting the mixture to 2-dimensional gel electrophoresis.

The isolated protein still contained a substantial amount of Gc globulin, identified by its neighboring position in the Anderson chart of human serum polypeptides. Antibody prepared with protein isolated from psoriatic serum and separated from Gc globulin by isoelectric focusing developed a single band against both psoriatic and normal serum in an Ouchterlony diffusion assay. Immunoelectrophoresis gave the same result. Displacement chromatography of a presumably normal serum yielded highly enriched fractions after the first pass that clearly contained the psoriasis-related protein, as identified by 2-dimensional gel electrophoresis.

Significance to Cancer Research (Objective 2, Approach 3)

Displacement chromatography of proteins, nucleic acids, etc., promises to be of value at any scale of operation and therefore has significance to all research that involves the isolation of such substances. The high capacity and convenience of these systems offer to expedite the recognition and isolation of minor protein components, such as regulating factors and marker proteins of interest in disease, when large enough samples are available for enrichment by these procedures. The development of a systematic, general procedure for the purification of nuclear proteins that are involved in the regulation of the transcription of genetic information would be of substantial significance to cancer research since cancer appears to be the result of defective control of this process.

Proposed Course of Research

Development of displacement systems for the separation of nuclear proteins will be continued, with special emphasis on fractionating the LMG proteins.

Displacement chromatography will be applied to the purification of enzymes and other proteins of interest to members of this Laboratory. Preliminary trials on tissue cytosols have been very promising.

The coarse prefractionation of human serum prior to 2-D electrophoresis will be developed further, using narrow-range CM-D, in the hope of providing a general approach in the search for marker proteins in disease that can be extended into a purification procedure for the markers thus identified.

Displacement chromatography will be applied to the isolation of serum and cytoplasmic factors mediating bone marrow response to inflammation in the guinea pig. Its possible application in the fractionation of cell populations and leukocyte granules will be explored, using appropriate adsorbent matrices.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 00945-08 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Factors Regulating the Synthesis of Collagen in Normal and Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Peterkofsky, Ph.D.	Research Chemist	LB NCI
OTHER:	M. Chojkier, M.D.	Expert Consultant	LB NCI
	U. Chauhan, Ph.D.	Visiting Fellow	LB NCI
	N. Blumenkrantz, Ph.D.	Visiting Scientist	LB NCI
	G. Majmudar, Ph.D.	Visiting Fellow	LB NCI
	L. Liotta, M.D.	Senior Investigator	LP NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.25	3.25	2.0

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying the role of ascorbic acid and neoplastic transformation in regulating collagen metabolism in animal cells. Deficiency of ascorbate in guinea pigs leads to decreased proline hydroxylation and defective collagen synthesis. In contrast, stationary cultures of L-929 cells do not require ascorbate for proline hydroxylation and contain a substance in the microsomal membrane which can effectively hydroxylate proline during procollagen synthesis in the rough endoplasmic reticulum.

PHS-6040
(Rev. 2-81)

Objectives: To elucidate the basic mechanisms of collagen synthesis and secretion and to investigate the factors which regulate these processes in normal and transformed cells.

Methods Employed:

1. Proteins of whole chick embryos, isolated bones of these embryos, or cell-free systems derived from these bones or cultured fibroblasts are labeled with ^{14}C-proline. The proteins are precipitated with trichloroacetic acid, redissolved and then assayed for radioactivity in collagen and noncollagenous proteins by a method involving specific digestion of collagen in a mixture of proteins using highly purified collagenase. This method was developed in our laboratory and is described in a previous report (1968-9). The relative rate of collagen synthesis can be calculated from data obtained by this method and the specific rate of synthesis in tissues or cells is calculated by determining the DNA content and expressing the rate as cpm/μg DNA. The proteins of the cell and medium fractions of cultured fibroblasts are analyzed separately in order to study secretion. Morphological changes in cultured cells are recorded by polaroid photomicroscopy.

2. To determine the level of proline hydroxylation in collagen of cells or tissues labeled with radioactive proline, collagenase digests are either hydrolyzed with 6N HCl and radioactive proline and hydroxyproline in the hydrolyzates are measured by a specific radioassay or a new dual labeled proline method is used which eliminates the necessity of hydrolysis. To measure the level of lysine hydroxylation, cells or tissues are labeled with radioactive lysine and hydrolyzed collagenase digests are chromatographed on Dowex-50 in 2N HCl, which separates lysine and hydroxylysine.

3. Prolyl and lysyl hydroxylases are measured by $^{3}H_2O$ release from ^{3}H-proline or ^{3}H-lysine labeled unhydroxylated collagen prepared by incubating chick embryo frontal bones with the labeled amino acid in the presence of the iron chelator α,α-dipyridyl, which inhibits hydroxylation.

4. Collagen types synthesized in cultured cells were determined by analysis of ^{14}C-or ^{3}H-proline labeled, denatured collagen using gel electrophoresis in sodium dodecyl sulfate-polyacrylamide slab gels. Disulfide bonds are detected by running samples with and without dithiothreitol and observing alterations in the positions of α chains. Radioactive proteins are detected by fluorography.

Cell lines used in these studies:

Chick embryo fibroblasts: prepared by digesting frontal bones from 15 day chick embryos with crude collagenase and culturing the released cells. Subcultures in the second to fifth passage are used.

L-929--a line of mouse embryo fibroblasts established about 30 years ago by use of a chemical carcinogen.

BALB-3T3--a contact inhibited line of mouse embryo fibroblasts isolated by Todaro and Aaronson and subclones of this line, P3 and 714 which are more

stringently contact-inhibited than the original culture and P13, which has lost density dependence.

Ki-3T3-234--a line established by Aaronson by transformation of BALB 3T3 cells with Kirsten sarcoma virus. These cells do not produce viral particles unless super-infected with murine leukemia virus.

Mo-3T3--BALB 3T3 cells transformed with Moloney sarcoma virus.
SV-3T3--BALB 3T3 cells transformed with SV-40 virus.
NQT-3T3-714--BALB 3T3 cells transformed with a chemical carcinogen, 4-Nitroquinoline-1-oxide.

Embryonic and adult human diploid fibroblasts purchased commercially.

BALB 3T3 subclones 3 and 714 were transformed with a temperature sensitive mutant of Kirsten sarcoma virus (KSV) containing Moloney leukemia virus (MLV) helper to give a mass infected and transformed line, tsKi(MLV)-3T3. Control lines infected with only MLV were also produced (MLV-3T3). A nonproducer subclone, tsKi-3T3-714 was derived from the transformed culture of 3T3-714.

Major Findings:

I. The role of ascorbic acid in collagen metabolism.

A. Effect on collagen levels in guinea pigs

Studies designed to analyze the mechanism by which collagen levels are decreased in scorbutic guinea pigs were continued. We have tested short-term cultures of guinea pig calvarial bone as a model. Guinea pigs were placed on a scorbutogenic diet, and after 2 weeks ascorbate had virtually disappeared from tissues. At this same time, the percentage of proline hydroxylation in collagen, measured in vitro, had decreased, but only by about 30%, and it remained at that level. When bones from scorbutic animals were labeled in the presence of ascorbate, hydroxylation returned to normal. In contrast, collagen production did not decrease significantly until after the second week and then decreased precipitously; by the fourth week, production was only about 30% that of the controls and in vitro addition of ascorbate did not increase collagen production to normal levels. These results suggest that the reduction in collagen production is a long-term effect of ascorbate deficiency, resulting either from secondary effects of underhydroxylation or from some other, as yet undescribed, effect of ascorbate on collagen metabolism.

B. Identification of an alternate reducing cofactor for prolyl hydroxylase.

In cultured cells, ascorbate deficiency does not result in complete underhydroxylation of proline. In some lines, such as Ki-MSV transformants and stationary phase L-929 cells, there is essentially complete hydroxylation in the absence of ascorbate. These cells do not synthesize ascorbate but they were found to contain another reducing cofactor for prolyl hydroxylase which was localized in the microsomal membrane. Purification of this substance is now in progress. It is a hydrophobic protein which can be

extracted from microsomes with Triton X-100. Further purification was achieved by gel filtration which yielded high and low molecular weight fractions. The latter is derived from the high molecular weight form and probably represents the active site. The low molecular weight fraction was further purified on P-2 gel and AG-1 columns.

II. Modification of the collagen phenotype by transformation

BALB 3T3 cells transformed by a variety of agents have a decreased relative rate of collagen synthesis and a change in the proportion of collagen types produced. The proportion of type III collagen produced by established lines of Kirsten or Moloney murine sarcoma virus (MSV) transformed cells was 5-6-fold greater than in several homogeneous subclones of untransformed cells, which produced mainly type I collagen. The collagen phenotype of an SV-40 transformant was unchanged while a nitroquinoline-1-oxide transformant produced almost no type I but instead produced two collagens normally associated with basement membranes, types IV and V.

By productively infecting and transforming cultures of homogeneous subclones of 3T3 with tsKi-MSV/MLV and thus eliminating cell selection, we showed that the changes in collagen synthesis and phenotype were due specifically to the sarcoma virus. The effect on the relative rate required functional src protein but the alteration in the collagen phenotype was retained at a nonpermissive temperature, suggesting several possible explanations for the phenotypic change:

1. Functional src protein is not involved in reactions leading to the altered collagen phenotype.

2. src protein initiates the change but a reaction involved is irreversible.

3. src protein is required but type III procollagen mRNA is long-lived.

The collagen produced by the nitroquinoline-1-oxide transformed 3T3 was identified as types IV and V by gel electrophoresis, cyanogen bromide peptide mapping and testing proteolysis with two enzymes specific for cleavage of type IV and V collagens. These experiments were carried out in collaboration with Dr. Lance Liotta of the Laboratory of Pathophysiology, NCI. Cell-free translation of RNA extracted from these cells verified that type I procollagen was not produced in these cells, although it is the major type produced by the parent cells.

III. Role of post-translational enzymes in procollagen biosynthesis

Differential detergent extraction of chick embryo bone microsomes has allowed us to localize prolyl hydroxylase in the cisternae and most of lysyl hydroxylase in the membranes. Localization of the enzymes which further modify hydroxylysine in procollagen to form the glucosylgalactosyl and galactosyl derivatives is under study. Preliminary experiments indicate that these enzymes are also intramembranous.

Proposed Course of Research:

I. Role of ascorbic acid in collagen metabolism

A. Experiments will be carried out to determine whether decreased synthesis or increased degradation of collagen can explain the decreased percentage of collagen produced by scorbutic bone cultures.

B. The low molecular weight form of the alternate reducing cofactor in L-929 cells will be identified. The high molecular weight form will be further purified by techniques suitable for hydrophobic proteins.

II. Alteration of collagen phenotype by transformation

The possible alternative mechanisms to explain the failure to revert the phenotype to normal in tsKiMSV transformed cells at the nonpermissive temperature will be examined. The half-lives of mRNAs for collagen types I and III will be determined by using inhibitors of RNA synthesis and analyzing the radioactive collagen produced during a pulse-label and also by measuring mRNA levels in a cell-free translation system.

III. Role of post-translational enzymes

We will study the role of the intrinsic membrane enzymes lysyl hydroxylase and the hydroxylysine glycosylating enzymes by means of a cell-free translation system using membrane-bound polysomes. The sequence of modification of lysine and proline residues will be determined.

Significance to Biomedical Research and the Program of the Institute:

Determining the precise mechanism by which vitamin C affects collagen synthesis should lead to a more accurate use of this vitamin. In addition, our studies with transformed cells indicate that, at least under some conditions, animal cells may be able to synthesize a substitute for the function of vitamin C in connective tissue.

The alteration of the collagen phenotype by transformation provides further information on biological changes caused by the carcinogenic agents as well as useful models for studying cellular differentiation.

Publications:

Chojkier, J., Peterkofsky, B., and Bateman, J.: A new method for determining the extent of proline hydroxylation by measuring changes in the ratio of [4-^{3}H]:[^{14}C]proline in collagenase digests. Anal. Biochem. 108:385-393, 1980.

Mata, J.M., Assad, R., and Peterkofsky, B.: An intramembranous reductant which participates in the proline hydroxylation reaction with intracisternal prolyl hydroxylase and unhydroxylated procollagen in isolated microsomes from L-929 cells. Arch. Biochem. Biophys. 206:93-104, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05202-14 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation, Fractionation, and Characterization of Native Nucleoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	O. Wesley McBride	Senior Surgeon	LB NCI
OTHER:	Jane Peterson	Senior Staff Fellow	LB NCI
	Usha Kasid	Fogarty Fellow	LB NCI

COOPERATING UNITS (if any)

Dr. Philip Leder, LMG, CH
Drs. David Swan & Stuart Aaronson, LCMB, NCI
Dr. Marcello Siniscalco, Sloan Kettering Institute

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	2.5	2.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop methods for gene transfer to mammalian cells and to use these techniques for gene mapping, analysis of gene expression, and cloning eukaryotic genes. DNA-mediated transformation provides a sensitive bioassay for dominant acting mammalian genes. A recombinant DNA library prepared from cells transformed with DNA from a heterologous species contains only a small fraction of the donor genome. The donor DNA sequences can be detected and isolated after nucleic acid hybridization with a labeled reiterated DNA probe prepared from donor DNA. The clone containing a specific gene then can be identified, using a transformation assay. We are using this method to clone the human tk and hprt genes. Chromosome-mediated gene transfer introduces a relatively large functional chromosome fragment into recipient cells. This procedure has been used for regional gene mapping and transferring closely linked, nonselectable genetic markers into cells. These large transferred genetic fragments also may contain adjacent regulatory sequences and the technique has potential for analysis of gene expression. We have used somatic cell hybridization to map human immunoglobulin genes and sequences homologous with MSV src genes to specific human chromosomes.

Objectives

Development of methods for gene transfer to mammalian cells and the use of these procedures for gene mapping, analysis of gene expression, and cloning eukaryotic genes.

Methods Employed

Tissue culture procedures, including plating in selective media, cloning, selection of cell mutants, and isolation of somatic cell hybrids after interspecific fusion of microcells with whole cells or cell/cell fusion. Metaphase chromosome isolation and purification by isopycnic and velocity sedimentation; gene transfer to mammalian cells by uptake of purified metaphase chromosomes or high molecular weight, eukaryotic DNA with subsequent isolation of biologically transformed colonies in selective media. Isolation and characterization of gene products by chromatography, electrophoresis, isoelectric focusing, and immunochemical procedures. Karyological analysis of hybrid cells and transformed cells by fluorescence, phase, and bright-field photomicroscopic techniques. Isolation of DNA and RNA, analysis of DNA reassociation kinetics, preparation of recombinant DNA, and cloning recombinant DNA in prokaryotes.

Major Findings

Our gene transfer studies fall into three categories:

DNA-mediated gene transfer: Transformation of eukaryotic cells with single copy genes, using whole genomic mammalian donor DNA, was first reported by Wigler, et al. (Cell 14: 725-731, 1978). We have used this technique extensively and demonstrated transfer of hypoxanthine phosphoribosyl transferase (hprt) and very rare cotransfer of galactokinase with a selectable thymidine kinase marker. This method provides a sensitive bioassay for dominant acting mammalian genes and it is useful for analysis of gene expression and for cloning eukaryotic genes.

There are currently several potential strategies for cloning eukaryotic genes and we have started cloning the human tk and hprt genes. Since no mammalian cells contain an abundant species of either tk or hprt gene transcripts, it is not possible to clone these genes by the use of appropriate cDNA probes. Previously cloned genes (bacterial Xgprt and HSV or chicken tk) do not exhibit sufficient homology with human hprt or tk to permit detection and isolation of these genes from human gene libraries. Our results also indicate that the "plasmid rescue" approach is not feasible. Digestion of high mol. wt. human and Chinese hamster DNA with various restriction endonucleases including Xba, Eco RI, Bgl II, Bam HI, Hind III, Bcl, Xho and Pst completely abolished both hprt and tk transforming activity, indicating recognition sites for all these endonucleases within these structural genes. The Kpn and Sma enzymes did not abolish tk activity but the DNA was not digested to a suitable size ($\leq$ 20 Kbp) to permit cloning.

Transformation assays after physically shearing human DNA to different lengths indicated that the human tk gene is smaller than 20 Kbp and probably smaller than 10 Kbp. (Sheared human DNA also retains hprt transforming activity but the size of the hprt gene has not been determined yet.) Therefore, DNA

has been isolated from mouse cells which were serially transformed with human tk by DNA transfer. These cells contain the selected donor tk gene but only a small fraction of the total human DNA sequence complexity. This DNA was sheared to 15-20 Kbp fragments without loss of tk transforming activity, size fractionated by velocity sedimentation, tailed with poly (dA), and annealed with Eco RI-cleaved, poly (dT)-tailed Charon 9 vector arms. The recombinant DNA will be incubated with λ phage packaging extract and used to transfect E. coli. The resulting "library" will be screened directly by transfer to nitrocellulose filters and hybridization with a human [^{32}P]-labeled repetitive DNA probe. Those rare plaques containing human DNA inserts will be isolated and purified from the library consisting predominantly of mouse recombinants. A plaque containing the intact human tk gene will be identified by transfer of plaque-purified recombinant phage DNA preparations to mouse LMTK$^-$ cells under selective conditions. The method is dependent upon the assumptions that most human DNA fragments of 20 Kbp length contain at least one hybridizable sequence of middle repetitive DNA and that these sequences can be demonstrated under conditions in which there is no detectable hybridization with mouse DNA sequences. We have verified both assumptions, using repetitive human DNA probes with mouse and human recombinant DNA libraries.

Chromosome-mediated gene transfer (CMGT): Functional genes can be transferred to mammalian cells by incubation with metaphase chromosomes. Only fragments of donor chromosomes are retained in recipient cells but closely linked genes are cotransferred at a relatively high frequency. Cotransfer of genes distantly linked on a chromosome, as well as cytologically detectable donor fragments, are observed at a lower frequency (i.e. ~ 10%). The transferred fragments can be replicated in recipient cells but ultimately they are integrated into the chromosomal DNA of the host cell. Our recent studies using nucleic acid hybridization analysis have provided quantitation of the amount of transferred DNA and it ranges from <2% to about 20% of the human X chromosome (i.e. <0.1% to 1% of the haploid genome). Our evidence, based on both nucleic acid hybridization analysis and the frequency of cotransfer of linked genes, indicates that much larger fragments of DNA are transferred by chromosome than by DNA-mediated gene transfer. Thus, CMGT has potential advantages for studying gene regulation and for obtaining cotransfer of syntenic nonselectable markers.

Preliminary studies suggest that it may be possible to transfer genes to mouse erythroleukemia cells by CMGT; enzyme characterization is in progress to exclude reversion as the mechanism for survival of recipient cells. This system would permit studying regulation of genes in the human β-globin gene cluster by CMGT.

We have also treated chromosomes with DNA-staining fluorochromes and determined the effect on chromosome uptake and donor gene expression. Pretreatment of chromosomes with the AT-specific fluorochrome Hoechst 33258 (2.5μg/ml) did not diminish the frequency of tk transfer to LMTK$^-$ cells. Studies are in progress to determine whether Hoechst 33258 treatment altered the average size of the transferred fragment. In contrast, chromosomes with bound GC-specific fluorochrome, chromomycin A3 (80μg/ml), were very toxic to recipient cells (<1% survival) and no tk transfer was observed.

A group of mouse A9 transformants expressing human hprt have been analyzed for expression of an X-linked human surface antigen in collaboration with Dr. Marcello Siniscalco of the Sloan Kettering Institute. The human X chromosomal DNA content of each transformant was determined by nucleic acid hybridization analysis. Preliminary results indicate that four of the transformants containing a very small amount of human X chromosomal DNA ($\leq$ 2%) do not express the antigen, whereas 10 other transformants contain larger amounts of X chromosomal DNA and also express this antigen.

Somatic cell hybridization: This has been a valuable technique for gene mapping in many laboratories. We have isolated and analyzed a panel of 25 independent human X rodent (both Chinese hamster and mouse) hybrid clones segregating human chromosomes. The specific human chromosomes present in each line have been determined by isozyme analysis, using 44 different markers which were previously assigned to the 22 different human antosomes and the X chromosome. Assignments have been confirmed by karyological analysis in some cases. This panel permits assignment of any gene to a specific human chromosome. The total content of different human chromosomes varies from one to 17 among the various hybrid lines. DNA has been isolated from each line and "blots" were prepared by Dr. David Swan after restriction endonuclease digestion and agarose gel electrophoresis. These reagents are currently being used to map human immunoglobulin constant region genes in collaboration with Drs. David Swan and Phil Leder and to map human genes homologous with the Abelson and Moloney MSV src genes in collaboration with Dr. Stuart Aaronson. The Kappa, Lambda and μ heavy chain constant region immunoglobulin genes and the MSV src gene all map on different human chromosomes. We have determined that the K and λ constant region genes are located on human chromosomes 2 and 22, respectively. Subclones have been isolated from appropriate hybrid lines after further loss of specific human chromosomes of interest. Analysis of these clones will permit confirmation of the gene assignments.

Proposed Course of Research

The human tk and hprt genes will be cloned by the "reiterated human DNA probe approach", which is in progress. If there is sufficient homology between the human and Chinese hamster tk genes, the hamster tk gene will also be cloned from a hamster recombinant DNA library. This may permit cloning the nonselectable galactokinase gene from our transformed cell line containing the transferred Chinese hamster tk and galK genes in close linkage. The cloned human tk and hprt genes will be used to study the copy number, location (free or integrated), and sites of integration of these genes in phenotypically stable and unstable transformed cells resulting from both chromosome- and DNA-mediated gene tranfer. They will also be used for sequence studies and comparison with previously cloned eukaryotic genes. Most importantly, availability of a human hprt probe will permit analysis of the human genetic disease, Lesch-Nyhan's Disease, at the DNA level as well as direct comparison of this gene on the functionally active and inactive X chromosomes in human female cells.

Efforts will be made to transfer the human non-α-globin gene cluster to mouse erythroleukemia cells by chromosome-mediated gene transfer. These studies in collaboration with Dr. French Anderson should permit the analysis of expression of developmentally regulated genes.

A panel of human X rodent somatic cell hybrids and subclones will be used to complete the mapping of human immunoglobulin constant and variable region genes and human genes having sequence homology with MSV src genes. Other human genes will be mapped as nucleic acid probes become available. Hybrid lines containing chromosome breaks and translocations will be used to determine the distance between specific immunoglobulin constant region and variable region genes.

Significance to Cancer Research (Objective 2, Approach 3)

Chromosome- and DNA-mediated gene transfer and somatic cell hybridization are important methods for chromosomal and subchromosomal regional mapping of normal and oncogenic genes. These gene transfer techniques also provide methods for the analysis of differentiated, developmentally regulated, and neoplastic gene expression.

Publications

(1) Athwal, R.S. and McBride, O.W.: Chromosome-mediated gene transfer and microcell hybridization. In Genetic Improvement of Crops: Emergent Techniques. (I. Rubenstein, B. Gengenbach, R.L. Phillips, and C.E. Green, eds.) U. Minn Press., Minneapolis, Minn., 1980.

(2) McBride, O.W. and Peterson, J.L.: Chromosome-mediated gene transfer in mammalian cells. Ann. Rev. Genetics 14: 321-345, 1980.

(3) Olsen, A.S., McBride, O.W. and Moore, D.E.: Number and size of human X chromosome fragments transferred to mouse cells by chromsome-mediated gene transfer. Mol. Cell. Biol., 1: 439-448, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05203-13 LB

PERIOD COVERED
October 1, 1980 to September 30,1981

TITLE OF PROJECT (80 characters or less)
Immunochemical Purification and Characterization of Immunocytes and Their Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.G. Mage	Immunochemist	LB NCI
OTHER:	L.L McHugh	Biologist	LB NCI

COOPERATING UNITS (if any)
Laboratory of Microbial Immunity, NIAID

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our goal is the development of cell separation methods for the specific isolation of immune cells, particularly for varieties of antigen-reactive cells (ARC) involved in cellular immune reactions, and for their subcellular fractionation in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. Populations of cells containing ARC are tested for binding to the cell surface antigens of target cells attached to insoluble supports. Separated populations are tested for cytotoxic effector cells (CTL) and their precursors, for activity in allograft rejection and graft-versus host reaction and in the mixed lymphocyte reaction. T cell subpopulations from thymus and spleen are also separated by and characterized with specific reagents such as peanut aggulutin and antibodies to the Lyt and CTL differentiation antigens. Surface molecules of target cells are isolated to test their binding to ARC.

PHS-6040
(Rev. 2-81)

Objectives

The goal of this project is the development of methods for the specific isolation of immune cells, particularly for different types of antigenreactive cells (ARC) involved in cellular immune reactions to alloantigens, to study the ontogeny of these cells, to study their roles, alone and in combination, in several in vitro and in vivo cellular immune reactions, and for their immunochemical characterization in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. In particular, we study the differentiation of cytotoxic effector cells (CTL) from precursors (CTLp) in thymus, the molecular reactions between immune cells and antigens bound to target cell surfaces, and the role of T cell subpopulations in in vivo cellular immune phenomona such as graft rejection and the graft-versus host reaction.

Methods Employed

ARC, obtained from normal mice or from mice immunized with allogeneic tumor cells or normal cells are incubated on surfaces coated with allogeneic or syngeneic target cells or on surfaces coated with antibodies to cell surface molecules. Graft-versus host activity (GVH) of nonadherent cells or of released adherent cells is measured by the Simonsen spleen weight assay in neonatal F1 mice. CTL activity is measured by ^{51}Cr release from target cells. Stimulation by alloantigen is measured by the mixed lymphocyte reaction and by generation of CTL in vitro.

T cell subpopulation from thymus and spleen are separated and characterized by their reactions with specific reagents such as peanut agglutinin and antibodies to the LyT and CTL (Rothstein et al. J. Immunol. 120: 209, 1978) differentiation antigens. Cell surface molecules of target cells are isolated to test their binding to ARC.

Major Findings

(1) Obligatory help for generation of CTL

Our development of a preparative non-lytic separation of $Lyt2^+$ and Lyt2 T cells has enabled us to examine directly the question of help for the generation of CTL. When highly purified $Lyt2^+$ responder spleen cells are put in primary mixed lymphocyte culture with semiallogeneic B cells as stimulators, no CTL are generated, despite the presence of CTL precursors in the $Lyt2^+$ population. However, if $Lyt2^-$ cells are added, then CTL are generated to the same extent as with an unfractionated responder cell population.

(2) The lymphokine "IL2" can substitute for $Lyt2^-$ cells in providing help to splenic CTL precursors in primary mixed lymphocyte culture

In experiments in collaboration with John Farrar of the Laboratory of Microbiology and Immunology, NIDR, we have found that in the absence of $Lyt2^-$ cells, but in the presence of IL2, CTL are generated from highly purified $Lyt2^+$ responder spleen cells in primary mixed lymphocyte cultures with semiallogeneic B cells.

Significance to Cancer Research (Objective 2, Approach 1)

The T cells that undergo maturation in the thymus are thought to be directly involved in anti-tumor immunity, by means of cell-mediated tumor rejection. Cytotoxic cell precursors,and helper and suppressor T cells are thought to undergo maturation in the thymus. Knowledge of the antigen-binding characteristics, differentiation antigens, and reactivities of immune cells at different stages of differentiation may help in developing specific purifications of various types of immune lymphocytes reactive against tumor antigens and in specific suppression of the graft versus host reaction, which is a serious iatrogenic complication of cancer therapy with bone marrow transplantation.

Proposed Course of Research

We plan to (1) continue to develop the general separation methodology based on specific binding of Ig-coated cells to anti-Ig-coated surfaces, (2) develop further separations of T cell subtypes, particulary CTLs, their precursors, helpers, and suppressors, and study their differentiation, (3) continue to study the contributions of these cells in vivo to immune reactions such as graft rejection, GVH reaction, and tumor rejection, and (4) study the macromolecules involved in the binding of CTL to target cells.

Publications

1. Mage, M.G.: Preparation of Fab Fragments from IgGs of different animal species. Methods in Enzymology, Vol. 70, p. 142, 1980.

2. Mage, M.G., Mathieson, B., Sharrow, S., McHugh, L., Hammerling, U., Kanellopoulos-Langevin, C., Brideau, Jr., D. and Thomas III, C.A.: Preparative nonlytic separation of $Lyt2^+$ and $Lyt2^-$ T lymphocytes, functional analyses of the separated cells and demonstration of synergy in graft-vs.-host reaction of $Lyt2^+$ and $Lyt2^-$ cells. Eur. J. Immunol. 11: 228, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05210-13 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cellular Controls over Growth and Inducible Processes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. B. Thompson	Chief, Biochemistry of Gene Expression Section	LB NCI
OTHER:	J. Harmon, Jr.	Staff Fellow	LB NCI
	F. Taub	Research Associate	LB NCI
	P. Earl	Cancer Expert	LB NCI
	R. Zawydiwski	Visiting Fellow	LB NCI
	J. Strobl	Postdoctoral Fellow, American Cancer Society	LB NCI
	B. P. Wagner	Animal Physiologist	LB NCI

COOPERATING UNITS (if any) P. Dannies, Yale Med. School; A. Kandutsch, Jackson Lab.; S.S. Simons, N. Lamontaigne, and L. Mercier, LC/A; H.J. Eisen, LBS/CH; J. DeLeo, CSL/DCRT; G. Crabtree, LP/C; M.C. Lippman, M/C.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Biochemistry of Gene Expression Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
11	9	2

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human glucocorticoid receptor has been purified and antiserum prepared to it. Human leukemic CEM cells have been propagated in nude mice and tested for steroid sensitivity. Dexamethasone mesylate has been shown to be a covalent affinity ligand for glucocorticoid receptors. Clones of wild-type CEM cells have been shown to vary in the onset of growth inhibition brought about by glucocorticoids. A glutamine-synthetase-inducible, but growth inhibitor-resistant CEM clone has been isolated. We have sequenced part of the rat growth hormone gene. A computerized image analysis method for screening arrays of cloned DNAs has been developed. We have found a cell line in which induction of tyrosine aminotransferase does not require full receptor occupancy.

PHS-6040
(Rev. 2-81)

Project Description

Objectives

Fundamental understanding of cellular controls of gene expression remains our prime objective. Studies of steroid hormone action at cellular and molecular levels are being pursued in an effort to achieve this goal. We remain interested in applying the fruits of basic research to clinical situations when it is feasible, and we have recently identified an unusual steroid with properties which may warrant its addition to the armamentarium of steroids in use clinically.

Specific objectives relevant to the above are as follows:

1) Objective one is to define controls over steroid responses in specific cell types. We employ several cell lines for this purpose: CEM cells, HTC cells, GH_3 cells, L cells, Fu55 cells, and IM9 cells. All of these but IM9 and Fu55 were described in last year's report. IM9 cells are a line of human lymphoblasts, rich in glucocorticoid receptors. Fu55 is a subclone of H35, a rat hepatoma line. The specific uses of these various cells are outlined in the several points below.

2) Objective two: To purify human glucocorticoid receptors from IM9 cells and produce antiserum to these receptors.

3) Objective three: To test deacylcortivazol and cortivazol as anti-leukemic-cell agents.

4) Objective four: To sequence the 5' regions of rat growth hormone and prolactin genes.

5) Objective five: To develop covalent affinity ligands for glucocorticoid receptors.

6) Objective six: To develop methods for and apply them to screening our library of double-stranded cDNA's prepared from steroid-treated rat liver.

7) Objective seven: To use our prolactin and growth hormone cDNA probes to examine restriction endonuclease digestion products from (rat) GH_3 x (mouse) L cell hybrids to establish: 1) the presence of both rat and mouse genes for these peptides in hybrids which do or do not express the gene products, and 2) if the genes are present, to see whether they have been altered in a major way.

8) Objective eight: To obtain fresh, unselected clones of CEM (human acute lymphoblastic leukemic) cells and compare their sensitivity to glucocorticoid and their receptor phenotypes with those of our clonal CEM-C7 sensitive line and its steroid-resistant selected subclones.

9) Objective nine: To compare the glucocorticoid responsiveness vs receptor occupancy of two rat hepatoma lines, HTC and Fu55, in both of which such steroids induce tyrosine aminotransferase.

Methods Employed

To those previously listed, we have added DNA sequencing by the method of Maxam and Gilbert, the "Southern" blot technique for analysis of specific DNA's, and computerized analysis of multiple, replica-plated bacterial clones carrying cloned mammalian DNA's.

Major Findings

This has been a very productive year, and we have been fortunate in achieving many of our goals.

Objective one: The exact controls over steroid hormone effect in specific cells remain unknown, however several new bits of data have been added to the picture.

We have found that, unlike reports concerning other viruses, the degree of methylation of mouse mammary tumor virus genes (in infected HTC cells) does not correlate with expression of the virus. We are examining the methylation state of growth hormone genes in inducible and non-inducible cells.

Other points follow in specific objectives.

Objective two: We have purified human glucocorticoid receptors from IM9 cells by the double-DNA-cellulose method of Eisen, and using this purified material as antigen have elicited antisera in two rabbits. IgG's from these sera bind to human glucocorticoid receptors.

Objective three: Using the nude mouse model system we developed last year, we have shown that CEM cells recultured after producing intrathecal tumors in these mice still show sensitivity to glucocorticoids in vitro. Mice injected with CEM cells and left untreated uniformly die. Preliminary data show that such mice when treated with cortivazol live significantly longer than controls treated with conventional glucocorticoids.

Objective four: We have sequenced approximately 675 bases of the 5' end of our rat growth hormone gene, from about -90 from the first exon continuously through to well within the second intron. We have begun to obtain the necessary cloned restriction fragments prior to sequencing the prolactin gene's 5' end.

Objective five: We have demonstrated that the new compound dexamethasone mesylate is a covalent affinity ligand for glucocorticoid receptors, as well as a longacting antiglucocorticoid in HTC cells.

Objective six: We have developed a computer-assisted image analysis method for comparative screening of large numbers of clones of bacteria containing mammalian DNA fragments in cloning vectors.

Objective seven: To pursue the question of why growth hormone and prolactin are neither expressed nor inducible in GH_3 x L cell hybrids, while they are both expressed and inducible in the parent GH_3 cell, we have examined both

parent and hybrid cells for the presence of growth hormone genes and mRNA. The non-expressing hybrids contain no translatable mRNA for growth hormone and none detectable by "Northern blot" analysis. Eight restriction endonucleases produce DNA digests which on Southern blot analysis show that the same pattern of L cell plus GH_3 cell growth hormone genes are present in both expressing and non-expressing hybrids. Similar analyses for prolactin are commencing. (We had shown previously that there is no translatable prolactin mRNA in the non-expressing hybrids.)

Objective eight: We have found that the majority of unselected CEM cell clones are sensitive to glucocorticoids, but that the onset of growth inhibition varies by as much as several days. We have derived steroid-resistant clones from one of these "wild-type" clones and found that their receptor phenotype appears to be similar to that we had obtained from our original sensitive clone, CEM-C7. This suggests that the phenotype we have described as predominating in the spontaneously resistant subclones is not unique to the offspring of CEM-C7. Finally, we have isolated a spontaneously resistant clone from the wild-type cells which contain apparently normal glucocorticoid receptors, as well as glucocorticoid-inducible glutamine synthetase. This clone thus shows that induction of this enzyme and cell killing are dissociable, and that the receptors the cells contain are functioning. It may be a candidate for the much sought-after cell with a lesion in the systems determining steroid responses beyond the receptor.

Objective nine: We have shown that full induction of TAT in Fu55 cells occurs at far less than full receptor occupancy. HTC cells, as most other cells and specific responses reported to date, are fully induced only by concentrations of steroid which fully occupy their receptors.

Proposed Course of Research

The successes we have had in several of the above lines of research offer great opportunities for the next few years.

We plan to characterize our anti-human glucocorticoid receptor antisera and their derivative purified IgG's with respect to species specificity and titer. We intend to use dexamethasone-mesylate labeled receptor to provide final proof of the specificity of interaction. We shall make antibody columns, use them to provide further purification of dexamethasone mesylate-labeled receptors, and use this receptor for such things as immunizing mice, preparatory to developing monoclonal antibodies by the "hybridoma" method. We shall attempt to accumulate enough pure receptor for partial sequence analysis.

We will try to use dexamethasone mesylate to radiolabel the receptors from steroid resistant (r^{actl}) CEM-C7 subclones and then submit the labeled receptors to 2-D gel analysis, to try to better define the physical basis for their abnormal behavior. If this methodology appears promising, we will seek to extend it to clinical material.

We hope to finish documenting that CEM cells in nude mice represent a good in vivo model system for leukemia therapy and especially to show that cortivazol holds promise for human trials.

We intend to complete our restriction endonuclease analyses of prolactin genes in GH_3 x L cell hybrids.

We will begin to explore microinjection of DNA's and proteins into GH_3, L, and GH_3 x L cells. Specifically, we intend to see whether: 1) injection of large quantities of growth hormone and/or prolactin DNA will overcome the L-cell-imposed extinction of their expression and 2) injection of L cell protein fractions will shut off expression of growth hormone and prolactin in GH_3 cells acutely and/or chronically.

We intend to set up a cell-free transcription system, based on that of Manley et al. and to try to obtain specific transcription of growth hormone and prolactin genes.

We want to use our computer-assisted technology to screen our library of induced rat liver cDNA clones, seeking minor as well as major increases or decreases.

We will attempt to map the rat prolactin and growth hormone genes to chromosomes, using flow microfluorometric chromosome sorting, followed by DNA extraction, endonuclease digestion and "Southern" blot analysis.

We will try to study dominance/recessiveness relationships and complementation by somatic cell hybridization experiments between various types of steroid resistant CEM cells.

Publications:

Original Papers

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Olson, P.S., Thompson, E.B., and Granner, D.K.: Regulation of hepatoma tissue culture cell tyrosine aminotransferase messenger ribonucleic acid by dexamethasone. Biochemistry 19: 1705-1711, 1980.

Schmidt, T.J., Harmon, J.M., and Thompson, E.B.: 'Activation-labile' glucocorticoid receptor complexes of a steroid resistant variant of CEM-C7 human lymphoid cells. Nature 286: 507-510, 1980.

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Allegra, J.C., Lippman, M.E., Thompson, E.B., and Simon, R.: The influence of steroid hormone receptors on response to chemotherapy in patients with metastatic breast cancer. In Steroid Receptors and the Management of Cancer, Vol. I, Thompson, E.B. and Lippman, M.E. (eds.), CRC Press, Boca Raton, FL, 1979, pp. 57-66.

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Thompson, E.B., Harmon, J.M., Norman, M.R., and Schmidt, T.J.: Glucocorticoid actions in a human acute lymphoblastic leukemia, T-cell line: A model system for understanding steroid therapy. In Hormones and Cancer, Iacobelli, S. et al. (eds.), Raven Press, New York, NY, 1980, pp. 89-98.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 05214-10 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

DNA Synthesis in Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Wilson	Medical Officer	LB NCI
	P. Becerra	Visiting Fellow	LB NCI
	A. Hazra	Visiting Fellow (11 mo.)	LB NCI
	S. Detera	Visiting Fellow (4 mo.)	LB NCI
	E. Karawya	Visiting Fellow (3 mo.)	LB NCI

COOPERATING UNITS (if any)

John Minna, NCI, VAMD

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Biosynthesis Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have examined the mechanism of E. coli DNA polymerase 1 large fragment using simplified reaction systems containing either $(dT)_{800}$ or $(dA)_{800}$ as template. This DNA polymerase was capable of highly processive DNA synthesis, incorporating several hundred dNMP residues per cycle of binding to the template, incorporation, and termination. We found that termination at any given product chain-length was markedly increased by the presence of higher concentrations of the template·primer complex. This and results of steady-state kinetic and enzyme/template binding studies suggested that the polymerase has more than one functional site for interaction with polynucleotides. Tryptic peptide mapping of putative DNA polymerase α subunits indicated that there is sequence homology between higher M_r (110,000) and lower M_r (50,000-65,000) polypeptides found in homogeneous preparations of the enzyme from calf thymus. A number of hybridoma clonal lines have been generated that appear to be producing monoclonal antibody to calf thymus α-polymerase.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: The ultimate objective of this research program is to understand mechanisms of DNA synthesis in mammalian cells. Our approach is the examination of DNA replication in vitro using purified DNA polymerases and other required proteins.

It is anticipated that these studies on the properties and specificities of the purified DNA replication proteins will, in conjunction with results from other workers, help answer important questions about mechanisms of DNA replication in the cell.

Methods Employed: Procedures have been developed for purification of DNA polymerases and DNA binding proteins from mouse and other tissues. These procedures involve subcellular fractionation, various types of ionic separation, gel filtration, and affinity chromatography, and assays for DNA polymerase activities using a variety of reaction conditions. Cell fractionation procedures and characterizations of reaction products were performed using conventional techniques of rate-zonal centrifugation, scintillation spectrophotometry, thin-layer chromatography, and gel electrophoresis.

Major Findings:

1. Mechanism of DNA Polymerase

a. Homopolymer replication systems and measurement of precise product chain length have been used to elucidate the mechanism of Escherichia coli DNA polymerase 1 large fragment. During replication of $\overline{(dT)_{800}}$ or $\overline{(dA)_{800}}$, this DNA polymerase was capable of acting processively, catalyzing many dNMP incorporation events during each cycle of binding to the template·primer complex, incorporation, and termination. Nascent chain termination was evaluated statistically by computing the probability of termination after each dNMP addition. These values changed as the chainlength of the product increased. Thus, for the first 5 to 10 dNMP residues added to the primer, the termination probability declined with each dNMP addition; then, after about 15 dNMP residues had been added, termination probability increased exponentially with each successive dNMP addition. These results are interpreted in the context of a kinetic model that requires the existence of two synthesis phases during formation of each long product molecule.

Further results from steady-state kinetic and product size distribution analysis suggested that polynucleotides can interact with this enzyme at more than one functional site. Substrate kinetic studies using the $(dT)_{800}\cdot\overline{(rA)_{20}}$ template·primer system indicated an apparent Michaelis constant for the template·primer complex of ~ 2.5 µg/ml or 0.13 µM primer 3' ends. This template·primer complex also exerted hyperbolic substrate inhibitions on the initial rate of overall DNA synthesis (K_i=25 µg/ml). Product size analysis indicated that higher levels of template·primer complex, in the range of 20 to 30 µg/ml, markedly increased the chance that the enzyme would terminate

after any given dNMP incorporation. Results from a physiochemical study on quenching of intrinsic protein fluorescence by $(dT)_{800}\cdot(rA)_{20}$ were consistent with the presence of two binding sites on the enzyme with first-order binding constants of 1.5 µg/ml and 27 µg/ml, respectively. These observations indicate that polynucleotides can terminate synthesis by the DNA polymerase 1 large fragment and that this effect may occur through interaction at a secondary polynucleotide binding site on the enzyme.

b. We have developed an affinity labeling method for a DNA polymerase active site using pyridoxal phosphate. The aldehyde group of pyridoxal phosphate reacts with proteins to form a Schiff's base involving the epsilon-amino group of lysine residues, and by reduction with [^{3}H]sodium borohydride, this protein-pyridoxal phosphate linkage can be rendered covalent and radioactive. Because pyridoxal phosphate inhibition of several DNA polymerases is known to be competitive with dNTP, the pyridoxal phosphate/sodium borohydride reaction appears to be a way of affinity labeling a polymerase active site. We have now found that the E. coli DNA polymerase 1 large fragment can be made radioactive using this reaction, and evidence for active site specificity of some of the labeling was provided by the observation that dNTP blocked most of the tritium incorporation into the enzyme. Interestingly, the enzyme was labeled in the absence of a template·primer complex and some of this labeling was dNTP sensitive. This finding may not be consistent with the current kinetic model for the mechanism of this enzyme; this model requires binding to the template·primer substrate before the kinetically significant binding site for dNTP is created.

2. Structure of Mammalian DNA Polymerase α

a. In collaboration with Waltraud Albert and Friedrich Grummt of the Institute of Biochemistry, University of Würzburg, G.F.R., the subunit structure of mammalian DNA polymerase α is being further investigated. Published studies by us and D. Korn and coworkers, Stanford University, had sugested that α-polymerases from calf thymus, mouse myeloma, and human KB cells were multi-subunit enzymes with individual subunits in the molecular weight range of 50,000 to 65,000. However, I.R. Lehman and coworkers, Stanford University, recently reported that D. melanogaster α-polymerase could be purified to a stage where the enzyme preparation contained just one polypeptide of ~ 145,000 daltons. This finding lead Lehman and coworkers to suggest that proteolytic digestion during isolation may have been responsible for the abundance of lower molecular weight polypeptides observed in the preparations of homogeneous mammalian α-polymerase.

During the past year, we have obtained homogeneous preparations of calf thymus α-polymerase that have an abundant polypeptide of apparent M_r = 110,000, in addition to the 50,000 to 65,000 polypeptides found previously. This 110,000 polypeptide represents ~ 50% of total dye stained protein visible in SDS-polyacrylamide gel analysis. Our tryptic peptide analysis has now indicated that this 110,000-M_r polypeptide shares homology with at least one of the lower molecular weight polypeptides. Other experiments indicate that the 110,000-M_r polypeptide probably does not represent simple aggregation of the lower molecular weight polypeptides. These results permit the conclusion that at least one species of the α-polymerase in calf thymus contains an associated 110,000-M_r polypeptide and that this polypeptide shares

primary structure homology with some of the 50,000 to 65,000-M_r polypeptides. Friedrich Grummt and his associates have found recently that the 110,000-M_r polypeptide has the ability to catalyze DNA synthesis, even when the lower M_r polypeptides are not present. Thus, considerable progress has been made toward clarifying the structure of calf α-polymerase, since we now know that the earlier model for the structure of the enzyme is either not complete or not correct.

b. In a related project, rat monoclonal antibodies to calf thymus α-polymerase have been produced. This work is being conducted in collaboration with John Minna, NCI. Rats were injected periodically with 5 μg each of homogeneous α-polymerase. The animal with the highest titer of precipitating antibody against α-polymerase was eventually selected, and 1400 primary rat x mouse myeloma hybridoma "clones" were prepared and grown in culture for three weeks. The culture fluid from ninety-seven of these "clones" was found to contain α-polymerase specific antibody as revealed by an ^{125}I protein A sandwich assay using immobilized α-polymerase. After subcloning, 36 of the resulting hybridoma lines were selected for further study. All of these clones produced relatively large amounts of antibody capable of high affinity binding to α-polymerase, but not to control proteins, such as serum albumin.

Strong neutralization of DNA polymerase activity in vitro could not be demonstrated with antibodies from any of the 36 clones or with the serum from the immunized rat. Two of the 36 clones produced antibodies that were weakly neutralizing, however. We anticipate that these antibodies will be useful for the rapid purification of α-polymerase, as well as for investigation of other problems concerning DNA polymerase biochemistry.

3. Characterization of DNA Exonuclease Activities

We have applied a published method of polyacrylamide gel electrophoresis to the analysis of oligonucleotide digestion products formed by DNA exonucleases. Among other findings, this approach has lead to the identification of two new DNA exonucleases in a fraction of mouse myeloma ssDNA binding proteins. These enzymes may be analogous to two DNA exonucleases (DNases VII and VIII) recently found in an extract from human tissue by Hollis and Grossman (J. Biol. Chem. (1981) in press). The mouse enzymes have molecular weights in the range of 25,000 to 40,000, require divalent cation for activity and are much more active with ssDNA substrates than with dsDNA substrates. One enzyme acts distributively, releasing 5' dNMP in the 3' → 5' direction. The other enzyme is processive. Its polarity of degradation is probably 5' → 3'; the main products released are $(pdN)_2$ and $(pdN)_3$, and the limit digestion products, corresponding to the 3' end of the substrate, are $(pdN)_4$ and $(pdN)_5$.

4. DNA Enzymes in Vaccinia Virus Infected HeLa Cells

We have continued an analysis of two viral induced enzymes described in an earlier report. One of these is a highly purified DNA polymerase, and the other is a partially purified protein capable of stimulating replication by the polymerase of a model single-stranded circular template, fdDNA. Without

this stimulating protein, the viral induced DNA polymerase is relatively inactive with fdDNA as template. This was expected since the system did not contain a substrate polynucleotide upon which the enzyme could initiate. We had shown earlier that preincubation of fdDNA with this stimulating protein and 4 rNTPs resulted in formation of short RNA molecules; about 5% of this RNA became attached to the DNA formed during a subsequent or second stage incubation with the DNA polymerase and 4 dNTPs. We have now shown by "^{32}P transfer" experiments that covalent RNA-DNA "joints" are formed during the second stage DNA synthesis reaction and that the predominant linkage is rA-dG. This result is consistent with a model in which the stimulating protein forms short polynucleotides, some of which can function as an initiator substrate for the DNA polymerase.

5. Regulation of DNA Polymerase Alpha in Monkey Cells in Culture

In collaboration with E.L. Kuff, we have continued an examination of levels of α-polymerase activity in contact inhibited monkey cells in culture. The results of this study are still preliminary, and we anticipate detailed description of findings in a later report.

Significance to Cancer Research: Detailed knowledge of the mechanisms of DNA synthesis is vital to our understanding the molecular biology of neoplasia. Many approaches are being used to investigate DNA synthesis in normal and neoplastic cells, and advances on all levels will prove useful in preventing, treating, and ultimately controlling cancer. Activity of DNA synthesizing proteins is necessary for maintenance of a rapid rate of cell division, and in some cases there is evidence that these proteins may play an important role in the development of the neoplastic state.

Proposed Course of Research:

1. To further characterize DNA replication proteins from mouse and monkey cells and from vaccinia virus-infected cells.
2. To further study the enzymatic mechanism of DNA polymerases and the properties of single-stranded DNA specific binding proteins of interest.
3. To investigate activity of purified DNA replication proteins in vitro using as template either a single-stranded closed circular viral DNA, viral replication intermediates, homopolymer DNA, or vaccinia virus DNA.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05231-07 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Subunit Interactions in Enzyme Chemistry and Cellular Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.B. Klee	Chief, Macromolecular Interactions Section	LB NCI
Others:	J. Haiech	Visiting Fellow	LB NCI
	M. Oldewurtel	Staff Fellow	LB NCI
	A. Rhoads	Guest Worker	LB NCI
	M. Epstein	Visiting Associate	LB NCI
	M. Krinks	Chemist	LB NCI
	J. Williams	Physical Science Technician	LB NCI

COOPERATING UNITS (if any) Dr. E. Neer, Harvard University; Dr. J. Schiloach, NIAMDD; Dr. D. Takemoto, Kansas University; Mr. Richard Feldman, CR-CCB; Dr. R. Adelstein, Cardiology Branch, NHLBI; Dr. P. Cohen, University of Dundee, Scotland; Dr. D. Hathaway, Indiana University.

LAB/BRANCH
Laboratory of Biochemistry

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.7	5.3	0.4

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The interaction of calmodulin with several of its target proteins including cyclic nucleotide phosphodiesterase, adenylate cyclase, myosin light chain kinase, phosphorylase kinase, cAMP dependent protein kinase, calcineurin and troponin I has been studied in order to understand the mechanism of regulation of Ca^{2+}-dependent cellular processes by this protein. At physiological concentrations of KCl binding of Ca^{2+} to its specific sites on calmodulin generates stepwise conformational transitions. The different Ca^{2+}-dependent interactions of calmodulin with its several protein targets allow calmodulin to effect a specific kinetic regulation of the Ca^{2+} signal. In contrast to phosphorylase kinase which interacts with calmodulin in the absence of Ca^{2+}, cAMP phosphodiesterase needs Ca^{2+} for interaction. This enzyme exhibits a highly cooperative Ca^{2+} activation and different degrees of Ca^{2+} occupancy are needed for interaction and activation. Enzyme activity can be modulated by factors which alter either calmodulin or Ca^{2+} levels. A third regulatory mechanism involves the interaction of the two second messengers, Ca^{2+} and cAMP. These are closely linked at the levels of phosphorylation of the target proteins, regulation of cyclic nucleotide and Ca2+ concentrations and also by virtue of interactions between the regulatory subunit of protein kinase and calmodulin.

Project Description:

Objectives:

To study the functional role of subunits and protein-protein interactions. The system under investigation is the Ca^{2+}-dependent regulation of enzymes mediated by calmodulin. Emphasis will be on cyclic nucleotide phosphodiesterase, enzymes regulating protein phosphorylation and their modulation by inhibitory proteins such as calcineurin and by the other second messenger, cAMP.

Methods Employed:

The project involves the study of properties of proteins, purified and analyzed by the usual techniques of protein isolation, including many types of chromatography and electrophoresis. The enzymes are studied by examination of their optical, hydrodynamic, and kinetic properties as well as by suitable chemical measurements and modifications. The role of these proteins in cellular regulation will be studied in tissue cultured cells.

Polynucleotide Phosphorylase:

Studies on the subunit structure of M. luteus polynucleotide phosphodiesterase and on the mechanism of the "de novo" synthesis of long polynucleotides catalyzed by the primer-independent form of the enzyme have been terminated.

Regulation of Cyclic Nucleotide Phosphodiesterase:

The Ca^{2+}-dependent regulation of several cellular processes is mediated by the ubiquitous, intracellular Ca^{2+}-binding protein, calmodulin. Regulation of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase by calmodulin is used as a model system to elucidate the mechanism of action of calmodulin.

The stimulus-response coupling mediated by Ca^{2+} involves several successive steps: (1) transitory increase in intracellular Ca^{2+} from 10^{-7} to 10^{-6}-10^{-5}M, (2) interaction of Ca^{2+} with the Ca^{2+} receptor, calmodulin, (3) interaction of the activated calmodulin·Ca^{2+} complex with the various target proteins and (4) coordinated activation of several enzymatic reactions. We previously reported that Ca^{2+} binding to calmodulin occurs in a stepwise fashion and is affected by monovalent as well as divalent cations. The identity of one of the Ca^{2+} sites occupied sequentially by Ca^{2+} was tentatively assigned to be Site IV by M. Epstein on the basis of fluorescence energy transfer studies using Tb^{3+} as a ligand. Binding of two mol of Tb^{3+} or two mol of Ca^{2+} per mol of calmodulin induces an increased tyrosine fluorescence which reflects a change in the environment of both tyrosyl residues 99 and 138. Binding of an additional Tb^{3+} results in a subsequent quenching of the tyrosine fluorescence and in increased Tb^{3+} fluorescence. The tyrosyl residue involved in this apparent energy transfer has been identified as Tyr 99, since nitration of Tyr 99 significantly reduces

the Tb^{3+} fluorescence. This result is also compatible with a distance of 5Å, between Tyr 99 and the Ca^{2+} bound in the fourth Ca^{2+}-binding site, as calculated by computer fitting of the sequence of the COOH-terminal half of calmodulin (sites III and IV) to the coordinates of carp parvalbumin in collaboration with R. Feldman. Tyr 138, on the other hand, is located more than 10 Å away from sites III or IV. Assuming that Ca^{2+} and Tb^{3+} have similar relative affinities for the different Ca^{2+} sites, the third site to be occupied is site IV. This assignment should, however, be considered only as tentative since Tb^{3+} has a much higher affinity than Ca^{2+} for the cation-binding sites and although it is an activator of calmodulin-dependent phosphodiesterase at very low concentrations it is a strong inhibitor at concentrations above 10^{-7}M.

The stepwise binding of Ca^{2+} or Tb^{3+} is accompanied by the formation of different conformers according to the degree of site occupancy. Calmodulin can thereby translate a quantitative Ca^{2+} signal into different cellular responses if different enzymes recognize different conformers. M. Oldewurtel has studied the structure of calmodulin in solution using susceptibility to limited proteolysis as a structural probe in an effort to characterize the individual Ca^{2+}-calmodulin conformers. The fully liganded protein is relatively resistant to trypsin which cleaves at Lys 77 to yield the two halves of calmodulin each containing two functional Ca^{2+}-binding sites. In the absence of Ca^{2+}, the protein is readily digested by trypsin, each susceptible bond being cleaved at a different rate. Quantitative analysis of the resulting peptides by high pressure liquid chromatography identifies those regions of the protein which are less structured in the absence of Ca^{2+} than in its presence. The structure predictions based on the experimental observations are in good agreement with those predicted according to the method of Chou and Fasman. This combined approach may localize the major conformational changes which accompany the binding of Ca^{2+} and enable us to achieve one major goal, that is to identify the Ca^{2+}-dependent interacting site(s) of calmodulin for its target proteins.

A second part of our research project consists of the study of the interaction of calmodulin with its target proteins. It has been carried out in the laboratory by M. Krinks, J. Haiech and A. Rhoads who is on sabbatical leave from Howard University, as well as in collaborative projects with Dr. R.S. Adelstein (NHLBI), Dr. J. Shiloach (NIAMDD), Dr. P. Cohen (University of Dundee, Scotland), and Dr. E. Neer (Harvard Medical School). The Ca^{2+}-dependent cyclic nucleotide phosphodiesterase has been islolated from bovine brain, rat brain and bovine heart. The three proteins, although slightly different in M_r have very similar peptide maps, but are significantly different from bovine retina cGMP phosphodiesterase and the "low k_m", cAMP specific, cyclic nucleic nucleotide phosphodiesterase from liver (these comparative studies were carried out in collaboration with Dr. D. Takemoto from Kansas University). The calmodulin-dependent phosphodiesterase is composed of two identical subunits with M_r of 59,000. It is converted by limited proteolysis to an active Ca^{2+}- and calmodulin-independent enzyme which is monomeric and has a M_r of 36,000. The subunit polypeptide is therefore composed of two domains, a catalytic domain, and a calmodulin-binding domain which exerts a regulatory, inhibitory effect on the catalytic domain. Several other calmodulin regulated enzymes also interact with calmodulin through their catalytic subunit such as myosin light chain kinase and phosphorylase kinase. More recently in collaboration with E. Neer (Harvard

Medical School), we have also shown that stimulation of brain adenylate cyclase by calmodulin does not require the presence of the regulatory GTP-binding protein.

None of the enzymes studies to date can form ternary complexes with calmodulin (binding of one protein prevents binding of another protein). Although some of these proteins may share a common calmodulin binding domain, it is more likely that there are some differences in the interacting sites since the requirements for interaction are different for different target proteins. In contrast to most calmodulin-dependent enzymes, phosphorylase kinase interacts with calmodulin in the absence of Ca^{2+} but needs Ca^{2+} for activation. The Ca^{2+} dependence of activation is therefore identical to the Ca^{2+}-binding isotherm of calmodulin and activation should be fast, controlled by the diffusion rate of Ca^{2+}. On the other hand, phosphodiesterase which required three to four Ca^{2+} per mol of calmodulin for interaction as well as for activation exhibits a highly cooperative Ca^{2+}-dependent activation curve. The interaction and the activation of the enzyme may also require different degrees of Ca^{2+} occupancy since the deactivation upon removal of Ca^{2+} is very fast and results from partial removal of Ca^{2+} from the activated complex these different modes of interaction enable calmodulin to regulate different enzymes to different extents and at different rates.

We have now obtained evidence that calmodulin regulation of cytosolic enzymes can also be modulated by other intracellular Ca^{2+}-binding proteins such as calcineurin as well as by cAMP-dependent phosphorylations. Conversely calmodulin and Ca^{2+} can exert a control over cAMP-dependent phosphorylations by acting directly or indirectly at the level of the cAMP-dependent protein kinase. In brain tissues, a fraction of the Type II cAMP-dependent protein kinase interacts with calmodulin in a Ca^{2+}-dependent fashion. This interaction occurs at the level of the regulatory subunit of the kinase.

Significance to Biomedical Research and the Program of the Institute:

The proteins being studied are important examples of enzymes regulated by protein-protein interactions.

cAMP phosphodiesterase, is one of the two enzymes responsible for the control of cAMP levels, which are critical for the regulation of cell growth and differentiation. The potential role of calmodulin in mediating the cellular effects of Ca^{2+} and the functional state of contractile and cytoskeleton proteins is of obvious importance. This ubiquitous regulatory protein may also provide a link between cyclic nucleotide levels and Ca^{2+} regulation of cell functions. The ability of calmodulin to regulate a large number of biological functions represents a novel mechanism with great potential physiological importance.

Future Course of Research:

Calmodulin plays a unique role in the regulation of cellular processes mediated by cytosolic Ca^{2+}: that of a universal modulator of Ca^{2+} acting as a second messenger. It may also function as a coupling factor in the dual regulation of cellular processes by Ca^{2+} and cAMP. We will continue to study the structure of calmodulin in solution to characterize the multiple Ca^{2+} conformers responsible for multiple functions, and to identify the calmodulin interacting site(s) with its target proteins. We will attempt to isolate and characterize the interacting sites of the target proteins cyclic nucleotide phosphodiesterase and calcineurin. We will try to identify the link between the two second messengers, Ca^{2+} and cAMP, by studying the effect of calmodulin and Ca^{2+} on cAMP-dependent protein kinase and the effect of phosphorylation on the stimulation of phosphodiesterase by calmodulin. These studies will be carried out at the molecular level with purified and characterized proteins as well as at the cellular level with cells in tissue culture to correlate the "in vitro" observations with physiological events. Among the model systems in which the role of calmodulin and other Ca^{2+} binding proteins will be investigated are: The differentiation of neuroblastoma glioma hybrid NG-108-15 and in vitro transformation of cells.

Publications:

Haiech, J., Klee, C.B. and Demaille, J.G.: Effects of Different Cations on the Affinity of Calmodulin for Calcium: A Possible Model for the Specific Activation of Calmodulin-Stimulated Enzymes. Biochemistry in press, 1981.

Klee, C.B., Oldewurtel, M.D., Williams, J.F. and Lee, J.W.: Analysis of Ca^{2+}-Binding Proteins by High Performance Liquid Chromatography. Biochem. Internatl. 2: 485-493, 1981.

Goldhammer, A.R., Wolff, J., Cook, G.H., Berkovitz, S.A., Klee, C.B., Manclark, C.R. and Hewlett, E.L.: Protein Activators of Bordetella Pertussis Adenylate Cyclase are Calmodulin. Europ. J. Biochem. 115:605-609, 1981.

Picton, C., Klee, C.B. and Cohen, P.: Phosphorylase Kinase from Rabbit Skeletal Muscle. Identification of the Calmodulin Binding Subunits. Europ. J. Biochem. 111: 553-561, 1980.

Adelstein, R.S. and Klee, C.B.: Purification and Characterization of Smooth Muscle Myosin Light Chain Kinase. J. Biol. Chem., in press 1981.

Hathaway, D.R., Adelstein, R.S. and Klee, C.B.: Interaction of Calmodulin with Myosin Light Chain Kinase and cAMP-dependent Kinase in Bovine Brain. J. Biol. Chem., in press 1981.

Barbehenn, E.K., Craine, J.E., Chrambach, A. and Klee, C.B.: Characterization of Polynucleotide Phosphorylase from M. luteus and Isolation of the 13,000 Base Poly(A) Product of the Polymerization Reaction. J. Biol. Chem., in press 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05234-07 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Interrelations between the Genomes of Simian Virus 40 and Green Monkey Kidney Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. F. Singer	Chief, Nucleic Acid Enzymology Section	LB NCI
Other:	T. Lee	Research Chemist	LB NCI
	G. Grimaldi	Fogarty Visiting Fellow	LB NCI
	S. Lord	Staff Fellow	LB NCI
	C. Queen	Staff Fellow	LB NCI
	S. Segal	Expert	LB NCI

COOPERATING UNITS (if any)

Dr. Paul Berg, Department of Biochemistry, Stanford University Medical School

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.3	4.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A recombinant "library" of the DNA of the African green monkey (Cercopthicus aethiops) in a bacteriophage lambda vector was constructed. Using purified DNA fragments as probes, segments of monkey DNA homologous to the control region around the origin of replication of simian virus 40 (SV40) were isolated.

Three distinctly different monkey segments homologous to the control region of SV40 were detected and characterized by subcloning and primary nucleotide sequence determination. Each segment is only a few hundred base pairs long and contains multiple and disconnected sequences homologous to the control region of SV40. The number and arrangement of the homologous sequences in each of the three segments differs and is distinct from the arrangement in the virus. The segments include homology to SV40 DNA regions known to be involved in the initiation of viral DNA replication and the start of early transcription. Accordingly, one of the segments has been tested for its ability to replace the SV40 control region for these functions. Preliminary results indicate that the monkey segment contains transcription start sites.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

A range of interactions occur between the genomes of a permissive cell and an infecting virus. Using simian virus 40 (SV40) as a model, we have studied two aspects of such interactions. Previously we had investigated recombination between the two genomes by analyzing the structure of defective viral variants containing both viral and host DNA sequences. In order to understand better the mechanism of such recombination we are now investigating the nature of the recombining host sequences within the monkey genome itself, prior to their interaction with the virus. Another aspect of viral-host interaction concerns the use of common mechanisms for replication and for the regulation of gene expression. When SV40 enters a permissive monkey cell, it uses the host machinery for the initiation of early viral transcription and synthesis of early viral proteins. Subsequently, interaction of an early viral protein (T-antigen) with the viral genome is required for viral DNA replication; replication also involves the use of a number of host enzymes. T-antigen also causes an increase in the level of the host enzymes required for DNA replication and stimulates host DNA replication. These events clearly suggest that the viral and host DNAs might share similar sequences that are recognized by a variety of enzymes and regulatory proteins. Many of the DNA sequences that regulate transcription and replication in SV40 are contained within a non-coding control region of a few hundred base pairs surrounding the origin of replication. We searched for and found homologous sequences in the monkey genome and are interested in their structural and functional characterization.

Methods Employed:

Standard tissue culture procedures are used for the growth of monkey kidney cells and SV40. Procedures used for studying viral DNA synthesis include radioisotope tracer techniques, preparative and analytical ultracentrifugation, DNA-DNA hybridization both in solution and with DNA fixed to nitrocellulose filters, column chromatography, and selective precipitation of high molecular weight DNA. Extensive use is made of restriction endonucleases and both preparative and analytical gel electrophoresis for the analysis and preparation of DNA fragments. Specific enzymatic procedures are used for modification or isotopic labeling of DNA fragments. Primary nucleotide sequence determination of DNA fragments is carried out by direct DNA sequencing techniques using the chemical procedures (methylation and hydrazinolysis) introduced by Maxam and Gilbert. DNA fragments from SV40 as well as from defective variants are purified and prepared in μg quantities by recombinant DNA techniques using E. coli K12 cloning systems. Recombinant DNA techniques are also used for the construction and preparation of molecules containing specific portions of the SV40 genome covalently linked to E. coli plasmid DNA. Recombinant DNA techniques involving lambda bacteriophage as a vector are also used. The ability of cloned monkey segments to function as replication origins or sites for initiation of transcription is studied with special recombinant vectors designed for use in animal cells. Desired constructions are made in vitro and then amplified in E. coli before transfection of mammalian cells. All recombinant DNA experiments are carried out under conditions required by the NIH Guidelines for Recombinant DNA Research as approved by the NIH Biosafety Committee. All our work has been facilitated by extensive use of the NIH computer for storage and analysis of nucleotide sequence data.

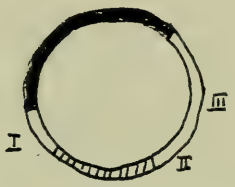
Major Findings:

The first step in our investigations involved the construction of a library of African green monkey liver DNA in a lambda bacteriophage vector. The insert fragments were obtained by partial digestion of monkey liver DNA with the EcoRI restriction endonuclease. Only a limited number of possible cleavages were permitted and multiple digests were pooled to provide a random assortment of fragments. Segments between 15 and 20 kilobase pairs (kbp) were purified by sucrose density gradient centrifugation in order to provide suitable insert sizes for the λ Charon 4A vector. Calculation indicated that ideally 2.5×10^5 phage should represent the entire monkey genome.

Approximately 2.5×10^5 phage plaques were screened by hybridization with a segment of SV40 DNA including the region around the origin of replication. The SV40 segment was first purified and amplified by molecular cloning in the E. coli plasmid vector pBR322. Six phage that hybridized with the SV40 segment under stringent conditions were detected and plaque purified. The monkey inserts were characterized by restriction endonuclease cleavage and gel electrophoresis and the size and location of the SV40-like segments were determined by Southern blotting of the gels and hybridization to the SV40 probe. These experiments indicated that the six phage represented only three different genomic segments (3 inserts were identical to one another as were 2 other inserts) and that in each case the homology to SV40 was restricted to a few hundred monkey base pairs. In addition, the SV40-monkey similarities were restricted to the region around the origin of replication of the virus. The three different SV40-like regions were then subcloned into pBR322 and the primary nucleotide sequence of each was determined.

All the segments contain multiple and disconnected sequences homologous to the region of SV40 directly surrounding the viral replication origin. The number and arrangement of the homologous sequences is different in the three segments. However, the segments have the following features in common: (1) each contains multiple copies of the sequence GGGCGGPuPu, which also appears six times near the origin of SV40, (2) each contains several strong homologies to the central dyad symmetry of SV40, (3) each contains a long internal repeat, as does the origin region of SV40.

Within the SV40 genome the GGGCGGPuPu repeats are known to be required for early transcription and to bind T-antigen. The central dyad symmetry region also binds T- antigen, is equivalent to the origin of replication, and a portion of it is the start site of early transcription. Accordingly, the ability of one of the monkey segments, that from clone 9, to replace the SV40 sequences for both viral DNA replication and in early transcription, has been tested. These experiments involve the use of a series of recombinant vectors constructed by Mulligan and Berg. The prototypical vector is pSV2.



Segments
pBR322
SV40
E. coli (xanthine) guanine-phosphoribosyl transferase
I: SV40 control region
II: SV40 splice site
III: SV40 polyadenylation site

To test whether the segment in clone 9 could serve as a replication the origin (I) was removed from the vector and replaced by the SV40-like region of clone 9. The new molecule was purified and amplified in E. coli, by means of the pBR322 sequences, and then transfected into monkey or mouse cells. There was no replication of the molecule in monkey cells, even when a source of T-antigen was supplied. Under these conditions the original pSV2 vector is replicated. Therefore we tentatively conclude that the monkey segment does not serve as an origin of replication. The ability of the monkey segment to serve as a "promotor" for transcription was measured in three ways. The first two depend on the monkey sequence permitting the expression of the bacterial enzyme, xanthine-guanine phosphoribosyl transferase. The gene for this enzyme is incorporated into pSV2 just downstream from the position of insertion of the monkey segment. When the SV40 control region is present at I, enzyme is produced as measured directly in transfected cells or by transformation of cells to grow in the presence inhibitor mycophenolic acid, an inhibitor of guanine nucleotide synthesis in mammalian cells, and xanthine, a substrate for the bacterial enzyme. The third method involved the isolation and mapping of RNA transcripts synthesized off the vector in transfected cells. By itself the monkey segment, inserted instead of the SV40 segment at I, does not support transcription or expression of the E. coli enzyme from the vector. However, in the presence of one particular portion of the SV40 control region, which by itself does not support transcription or expression, the monkey segment permits transcription and expression. This particular SV40 segment is defined by a 72 bp sequence repeated two times in the wild type genome. RNA transcripts synthesized off the vector are initiated at two positions within the sequence of clone 9. These results lead to a model of the SV40 promotor system that is consistent with results obtained recently now in several laboratories. The "promotor" is actually made up of several elements, each of which plays a role in modulating transcription. The monkey

segment appears to replace one such module and may also function in an analogous fashion within the monkey genome.

Previously we had investigated the structure of defective variants of SV40 that contain monkey DNA segments in place of viral DNA segments. One particular monkey segment, which recurred in a number of independent isolates of defective variants, was shown to represent a low copy number monkey genomic sequence. We have now carried out Southern blotting experiments using restriction endonuclease digests of monkey DNA and a probe consisting of the sequence from the variant, purified by molecular cloning in pBR322. These results indicate that there may be as many as four separate copies of this sequence in the monkey genome. We have tried to isolate these segments from the monkey library. However, only one phage containing a hybridizing sequence could be isolated. A second library was constructed in a different fashion, but yielded no phage that hybridized with the sequence of interest. The one phage from the EcoRI library has been characterized by restriction endonuclease mapping, hybridization and subcloning. The array of restriction endonuclease sites and within and flanking the sequence of interest indicates that it is not identical to the segment that occurs in the defective SV40 variants. We conclude that either the segment in the variants is derived from one of the other genomic copies of the sequence or else that extensive rearrangements occurred during the formation of the variant structure.

Significance to Cancer Research:

SV40 is an oncogenic virus. Oncogenicity appears to depend first on recombination of the viral genome with that of susceptible host cells, resulting in insertion of the virus into the genome of transformed cells, and then on the expression of viral genes in the transformed cells. Our studies deal with the interactions between the viral genome and the genome of a permissive host. These studies are pertinent to the nature and mechanism of viral-host DNA interaction, as well as to the events that lead to permissive rather than transforming infections. Further, it is now widely recognized that the genomes of RNA tumor viruses contain sequences homologous to normal host DNA sequences. Our experiments indicate that the same is true of DNA viruses, although in this case, regulatory sequences rather than coding sequences are involved.

Proposed Course of the Research:

During the coming year, the primary focus of our work will be the functional significance of the monkey DNA segments that are homologous to the SV40 control regions. The work that has been initiated with clone 9, will be extended to include the sequences in clone 7 and clone 5. The ability of the clone 5 and 7 sequences to support DNA replication will be investigated as will their possible role in transcription initiation. Although the expression of genes such as GPRTase will be used as an assay, the mapping of actual transcripts will be the main method. In addition, the binding of T-antigen to the monkey sequences will be investigated as well as the organization of the sequences within monkey chromatin.

Work on the monkey sequence that occurs in the variant will continue.

F-9 Murine teratocarcinoma stem cells do not support the growth of SV40 or polyoma nor the expression of the viral tumor antigens. One of the blocks to SV40 early gene expression in these cells seems to be at the level of RNA processing. Differentiation in vitro by retinoic acid overcomes the block and the cells begin to synthesize SV40 large T antigen and small t antigen at a normal rate. To understand the mechanisms involved in the processing of SV40 early mRNAs in stem versus differentiated cells, an E. coli plasmid which will provide unlimited amounts of unspliced SV40 early RNA (substrate) has been constructed. F-9 stem and differentiated cells as well as Hela cells will be fractionated, and the different fractions will be analyzed in vitro for RNA processing activity. The goal of these experiments is to develop an in vitro RNA processing system and study the differences between stem and differentiated cells.

The problem of viral host range is poorly understood. The availability of recombinant DNA techniques allows the construction of hybrid viruses of polyoma and SV40 in a way which will permit study of their regulatory signals in a variety of hosts. The construction of the hybrids will be done by exchanging known regulatory sequences from one virus to the other. After infection of murine and monkey cells viral gene expression will be followed at the level of transcription, DNA replication and protein synthesis.

Publications:

Winocour, E., Singer, M.F. and Kuff, E.: The rapid detection, isolation and amplification of host-substituted SV40 variants. Cold Spring Harbor Symp. Quant. Biol. 44: 621-628, 1980.

Singer, M.F. and Thayer, R.E.: The preferential replication of a class of host-substituted defective simian virus 40 variants at low temperature. J. Virol. 35: 141-149, 1980.

Singer, M.F., Kuff, E., Lee, T., McCutchan, T., Papamatheakis, J., Thayer, E. and Winocour, E.: A recurring SV40 variant containing monkey DNA sequences. In Scott, W.A., Werner, R., Joseph, D.R. and Schultz, J. (Eds): "Mobilization and Reassembly of Genetic Information", vol. 17, Academic Press, New York, 1980, pp. 303-316.

Papamatheakis, J., Kuff, E., Winocour, E. and Singer, M.F.: Structure of a newly isolated variant of simian virus 40 DNA containing monkey DNA and its similarity to previously isolated variants. J. Biol. Chem. 255: 8919-8927, 1980.

Papamatheakis, J., Lee, T.N.H., Thayer, R.E. and Singer, M.F.: Recurring defective variants of SV40 containing monkey DNA segments. J. Virol. 37: 295-306, 1981.

McCutchan, T.F. and Singer, M.F.: DNA sequences similar to those around the simian virus 40 origin of replication are present in the monkey genome. Proc. Natl. Acad. Sci. USA 78: 95-99, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05241-06 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies of SV40 Recombinants Containing Cellular and Viral Sequences

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. L. Kuff	Chief, Biosynthesis Section	LB NCI
OTHER:	T. Koch	Visiting Associate	Not NIH

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project was not active during the reporting period.

Project Description:

Objectives: To understand the process of recombination between SV40 DNA and the DNA of infected cells.

Methods Employed: See below.

Results: This project was not active during the reporting period.

Proposed Course of Research: During the coming fiscal year, we plan to use the transforming capacity of SV40 DNA as a selective marker for the introduction of specific mouse retroviral genes into cells of other species.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 05243-05 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

In Vitro Synthesis of Vitellogenin Messenger RNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. P. Mullinix	Formerly Research Chemist (As of November 1979, Assistant Director for Intramural Planning)	LB NCI
OTHER:	R. F. Goldberger	Formerly Medical Director (Chief) (As of September 1979, Deputy Director of Science, NIH)	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated.

Mullinix, K.P. and Goldberger, R.F.: Specific Transcription of Vitellogenin and Albumin RNA in Chromatin from the Livers of Normal and Estrogen-treated Roosters. In Delzio, G. and Brachet, J. (Eds.): Steroids and Their Mechanisms of Action in Non-mammalian Vertebrates. New York, Raven Press, 1980, pp. 161-170.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05244-04 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Organization of Repeated DNA Sequences in African Green Monkeys

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.F. Singer	Chief, Nucleic Acid Enzymology Section	LB NCI
Other:	T. Lee	Research Chemist	LB NCI
	R. Thayer	Chemist	LB NCI
	G. Grimaldi	Fogarty Visiting Fellow	LB NCI
	A. Maresca	Fogarty Visiting Fellow	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

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NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.3	4	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Individual monomer and dimer units of the highly reiterated cryptic satellite (called α-component) of African green monkey (Cercopithecus aethiops) were purified by molecular cloning and sequenced. The data indicate that the set of approximately 5 x 10^6, 172 base pair long sequences comprising α-component include a large number of related but slightly divergent sequences.

A group of monkey DNA segments about 17 kilobase pairs long and containing α-component sequences joined to other genomic DNA has been isolated from a library of green monkey DNA in λ-bacteriophage. Analysis of these sequences indicates that (1) domains of variant monomer units are grouped together, (2) the tandem repeats of α-component can be interrupted by other DNA sequences, (3) at least two classes of distinct sequences frequently occur joined to α-component and that these sequences themselves occur on the order of 10^3 to 10^4 times in the genome. The α-component sequences present on a single isolated monkey chromosome represent a special subset of the total.

The interspersed highly repeated 300 base pair long Alu family of sequences found by others in human DNA is conserved in African green monkey in sequence, dispersal and copy number.

Project Description:

Objectives:

The detailed structure and organization of the highly repeated DNA sequences in the genome of the African green monkey (Cercopithecus aethiops) are being studied. One set of sequences is referred to as the α-component and represents approximately 20 percent of the genome. Our earlier work showed that the several million copies of the 172 base pair long sequence that occur in the monkey genome constitute a set of tandemly repeated segments (172 base pairs in length) of very similar primary structure. A primary nucleotide sequence representing the most frequent nucleotide at each of the 172 positions in the set of α-component monomers was determined. Our goal now is to learn something about the number of different members of the set, the relative frequency of different members, the arrangement of the different members of the set relative to one another, and to other DNA sequences within the genome, the specificity of α-component sequences within a single chromosome and the way in which these sequences are packaged into chromatin structure. In recent years, it was discovered that mammalian genomes usually contain families of highly repeated DNA interspersed among unique DNA segments. The dispersed families are unrelated to satellites such as α-component. We are also investigating such sequences in the green monkey genome.

Methods Employed:

DNA is isolated from fresh frozen tissue and from cells grown in tissue culture and purified by standard procedures. Structural analysis includes the use of a variety of enzymes, including restriction endonucleases, direct DNA sequencing methods (chemical procedure of Maxam and Gilbert), radioisotope tracers, gel electrophoresis, and centrifugation. A variety of nucleic acid hybridization techniques, both in solution and on nitrocellulose are used. DNA fragments are purified and amplified by molecular cloning in E. coli K12 host-vector systems. All recombinant DNA experiments are carried out under conditions required by the NIH Guidelines for Recombinant DNA Research as approved by the NIH Biosafety Committee. Somatic cell hybrids are constructed by standard techniques.

Major Findings:

We have studied the primary sequence of a small group of monomer and dimer α-component repeat units chosen at random. Purified units were obtained by cloning, in the E. coli plasmid vector pBR322, α-component monomer and dimer fragments obtained from digestion of total African green monkey DNA with the restriction endonuclease HindIII. Recombinant clones were selected on the basis of their ability to hybridize with α-component. Selected recombinants were purified and the plasmids were isolated and characterized initially by restriction endonuclease digestion and then by primary nucleotide sequence analysis. This analysis indicated that all of the cloned α-component monomers and dimers had primary sequences different from that of the average sequence we had previously determined. The fact that in this work, as well as earlier work of our own and data on several more cloned repeat units obtained in other

laboratories, the 'average' monomer sequence has never been found, indicates that α-component contains a large number of different sequences and that the average sequence occurs rarely if at all. The total divergence from the average sequence, calculated from the sequenced units, is of the order of 3 percent. Further, the sequence data indicate little or no clustering of divergent sequence alterations; single base pair changes are spread throughout the 172 base pair sequence. These results are consistent with a pattern of random divergence. However, other results obtained previously as well as those described below, indicate a pattern of non-random divergence among α-component sequences. Thus, certain alterations in the average sequence, as measured by the presence of certain restriction endonuclease cleavage sites, occur more frequently than others. It seems probable that the observed non-random divergence reflects particular subgroups of repeat units and is superimposed on a generalized apparently random divergence. This interpretation is supported by the data described below. It is also consistent with the following model for the evolution of the highly repeated sequences: initially randomly diverged copies of the repeat unit are preferentially amplified and then undergo additional random mutation.

We wanted to study DNA segments in which α-component is joined to other DNA segments. For this purpose we constructed a library of the monkey genome in the bacteriophage lambda. Segments of the monkey genome between 15,000 and 20,000 base pairs long were generated by a process designed to randomize most of the monkey genome. These segments were then linked to the arms of the lambda vector charon 4A and packaged, in vitro, into phage particles. The phage were then amplified in a manner designed to enhance the likelihood that the final collection of phage would represent most of the original recombinants. The library was screened for plaques that hybridize with α-component. Approximately 0.7 percent of the plaques gave a strong hybridization signal with a ^{32}P-labeled α-component probe. On a random basis, and considering that about 15 to 20 percent of the total genome is α-component, it can be calculated that the expected percent would be of the order of 15. The discrepancy between what was observed and the calculated value is consistent with our assumption that the very long tandem stretches of α-component that contain no EcoRI site at all would not occur in the library; such stretches must include almost 90% of the total α-component in arrays containing 100 or more copies of the 172 base pair monomeric unit.

Sixteen phage were chosen at random from among those in the library that hybridized with α-component and have been characterized to varying extents. Restriction endonucleae digestion and Southern blotting experiments showed that the α-component monomer units cloned in the phage differ significantly from the average units characterized from bulk DNA. The differences include repeated occurrence of generally rare restriction endonuclease sites such as EcoRI and BamHI as well as the frequent absence of the HindIII site that occurs in the overwhelming majority of α-component monomer units. These observations permit us to conclude that the organization of α-component monomers includes long stretches of related variants, thus confirming the nonrandom aspect of divergence mentioned above.

At least 11 of the phage contain sequences other than α-component sequences and thus represent genomic junctions between satellite and non-satellite sequences. We have found that there is a much greater amount of sequence divergence in the α-component sequences joined to non-α-component, than that found in the average sequence characterized from bulk DNA. This finding is consistent with the predictions of the unequal crossing-over model for satellite evolution. Two groups of three phage each appeared, on the basis of restriction maps, to contain similarities in the non-α-component boundary sequences and these have been analyzed in more detail. One group, termed the Bam group, all showed a 1 kb BamHI restriction endonuclease fragment that does not hybridize with α-component. This fragment is at the boundary between the satellite and non-satellite sequences. The 1 kb BamHI fragments from two phages, λCaα2 and λCaα4, were purified by subcloning in pBR322. The two fragments cross-hybridize with one another and with the third phage, λCaα5 thus demonstrating that common boundary sequences do occur in these three monkey sequences. Southern blotting experiments using restricted monkey DNA and the cloned BamHI fragments as probes indicate that the sequence occurs several thousand times in the monkey genome. The second group, termed the Kpn group, also showed similar restriction endonuclease maps at the junction of α-component and non-α-component sequences. A 2.8 kilobase pair KpnI fragment was subcloned from one of these, λCaα6, and the sequence was shown to crosshybridize with the other two phage in the group, λCaα7 and λCaα11, but not to hybridize with α-component itself or with the 1 kb BamHI fragment. Like the BamHI fragment, sequences within the cloned Kpn fragment occur several thousand times in the monkey genome.

λCaα6 is of special interest because the non-α-component sequences are flanked on both sides by α-component. This observation demonstrates that satellite sequences can be interrupted by non-satellite sequences, an unexpected finding. At least one other phage in the group of 16 also appears to have an interrupted α-component. In this instance, λCaα9, the interruption is a copy of a segment homologous to the Alu family of interspersed repeated primate sequences.

The dominant family of interspersed (as distinguished from tandemly reiterated) repeated sequences in the human genome has been termed the Alu family. These 300 base pair long segments occur about 3×10^5 times in the human genome. We have found that more than 75% of the phage in the African green monkey recombinant library hybridize with a cloned human Alu sequence under stringent conditions. Detailed analysis of selected phage indicates that some contain more than one Alu sequence, with the average separataion of Alu-like sequences being about 5 kilobase pairs. Thus, both the sequence, copy number, and dispersion of the human Alu family is conserved in the monkey genome. Furthermore, at least 7 of the α-component phage described above also contain Alu sequences. Included in these are λCaα9, mentioned above, as well as λCaα6 and 7, also mentioned above.

Somatic cell hybrids were constructed between African green monkey kidney cells and mouse LB82 cells. The LB82 cells are mutant in thymidine kinase (TK^-) and hybrids were grown in HAT medium in order to select mouse cells containing the single monkey chromosome encoding thymidine kinase. Several such clones were isolated. The presence of the appropriate chromosome was confirmed by the

presence of the monkey galactose kinase enzyme in the hybrid clones (the galactose kinase gene is on the same chromosome as thymidine kinase in primates) and the presence of a single monkey chromosome was demonstrated by differential staining with Giemsa-11 and back selection in bromouridine. DNA was prepared from a cloned hybrid and compared with both monkey and mouse DNA by restriction endonuclease digestion, Southern blotting, and hybridization to α-component sequences. Mouse DNA did not hybridize with the monkey satellite. The results indicate that the hybrid contains a set of α-component sequences that are clearly different from bulk α-component isolated from monkey DNA. In particular, most of the α-component monomers associated with the isolated monkey chromosome contain a site for the restriction endonuclease HaeIII, a very rare site in bulk satellite. Thus we conclude that particular domains of α-component are associated with individual chromosomes.

Significance to Cancer Research:

Cancer research has suffered from a lack of basic knowledge about the eukaryote genome at the molecular level. Methods developed during the last few years have already demonstrated their power to deal with this vast and important unknown. The work we are doing is part of the broad effort to apply new approaches to the elucidation of complex genomes. In particular, we are concentrating on the surprisingly large amount of primate DNA included in the highly repeated sequences of yet unknown function. With increased understanding of DNA organization, especially in primates, existing information and new data on neoplastic disease may become more amenable to rigorous analysis and understanding.

Future Course of the Research:

(1) The structure, organization, frequency, and chromosomal location of the BamHI and KpnI boundary sequences will be investigated. We will also study the conservation of these sequences in the human genome.

(2) The detailed organization of α-component sequences in the isolated monkey chromosome will be studied. In addition, we will continue attempts to isolate other monkey chromosomes by analogous procedures.

Publications:

Thayer, R.E., Singer, M.F. and McCutchan, T.F.: Sequence relationships between single repeat units of highly reiterated African green monkey DNA. Nucleic Acids Res. 9: 169-181, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05246-04 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Organization and Expression of Genetic Information in Lower Eukaryotes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. A. Kramer	Senior Staff Fellow	LB NCI
Others:	N. J. Andersen	Chemist	LB NCI
	G. Thill	Chemist	LB NCI
	A. M. Musti	Fogarty Visiting Fellow	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project employs recombinant DNA techniques to isolate individual genes of yeast. These clones are then used in studies of the organization and expression of these genes at the molecular level.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

The goal of our research is to contribute to an understanding of the ways in which the genes of an organism are arranged and of the molecular mechanisms involved in the expression of genetic information. The yeast Saccharomyces cerevisiae was chosen as a model system for this project because extensive genetic studies have been carried out with yeast and because yeast has a relatively small genome. While yeast is a relatively simple eukaryotic organism, it has many molecular biological properties similar to those of higher organisms and not found in prokaryotes. Thus, yeast provides a relatively simple system for studying eukaryotic gene organization and expression at the molecular level.

Methods Employed:

Recombinant DNA techniques are being used to clone individual yeast genes or groups of genes. The structure of these genes is then determined by restriction enzyme mapping, DNA sequence analysis and RNA mapping using hybridization and electron microscopy. Fully characterized clones are employed as probes to study in vivo gene expression and as templates for in vitro experiments to reproduce in vivo results. Transcription products are analyzed by RNA sequence analysis, gel electrophoresis, hybridization and RNA mapping techniques.

Major Findings:

1) We have used differential plaque filter hybridization to screen a phage "library" of yeast DNA for genes induced by growth in low-phosphate medium. Two such genes on EcoRI fragments of 8 and 5 kb were obtained. The genes on the two cloned fragments were demonstrated to code for the peptides p60 and p56, respectively, that had previously been shown to be similar peptides that corresponded to yeast repressible acid phosphatase (APase).

The mRNA map positions on and the homologous regions between the two EcoRI fragments were determined by R-loop analysis, S1 nuclease mapping, gel blot hybridizations and heteroduplex analysis. A region of about 1.5 kb that codes for a phosphate repressible mRNA was identified on each fragment. The fragments are homologous to each other over a region of about 2 kb that includes the mRNA coding sequences. An additional region of weak homology to the mRNA coding sequences was detected on the 8 kb fragment. This probably corresponds to the gene for a constitutive APase, PHO3, that maps very close to the major repressible APase gene, PHO5.

S1 nuclease mapping and reverse transcriptase extension of a DNA primer on low-phosphate induced RNA were used to precisely map the 5'-end of the mRNA encoded by the major repressible acid phosphatase gene, PHO5. Several distinct termini over a 20 bp region were found. Sequence analysis of the 5'-flanking region of the PHO5 gene showed the termini to be centered about 35 bp upstream from the translation initiation codon and 65 bp downstream from a "TATA" box that fits perfectly with the consensus sequence. It is not yet known if these termini represent primary transcription products. The region in which the termini map is very A-rich, a characteristic of the 5'-flanking region immediately adjacent to the translation initiation codon in many yeast genes.

Several segments of the 8 kb EcoRI fragment that contain the entire PHO5 coding region have been inserted into an autonomously replicating yeast vector (YRp12) and used to transform a pho5 mutant. All the transformants including one that has only about 250 bp of yeast DNA upstream from the 5'-end of the mRNA, show induction of APase activity in low-Pi medium, although not to the same level as in wild-type strains. We have constructed deletion mutations in the plasmids to define the sequences required for regulation and expression of the PHO5 gene.

2) Since a strong homology has been shown among the primary structures of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme of many diverse organisms, we investigated if a chicken cDNA clone with part of the GAPDH gene had any detectable homology with the GAPDH genes of yeast. In yeast there are three GAPDH structural genes per haploid genome and each one is encoded on a different EcoRI restriction fragment. The chicken cDNA clone was used a probe in a gel blot hybridization with an EcoRI digest of yeast DNA. The three characteristic fragments with the yeast GAPDH genes were detected.

The chicken GAPDH probe was used to screen a phage library of yeast DNA and one of the three yeast GAPDH genes was isolated. The GAPDH sequences on this clone were used to re-screen the library and both of the other GAPDH genes was isolated. These clones were then used to precisely map the termini of the messages and to examine the relative levels of the transcripts of the different copies of the GAPDH gene.

Significance to Cancer Research:

An understanding of the molecular mechanisms involved in gene expression will be necessary to understand the processes by which a cell loses its genetic regulation and becomes cancerous. This project is designed to determine some of the normal mechanisms of gene expression and regulation as a basis for identifying the changes which have occurred at the molecular level in malignant cells. Knowledge of these changes should provide valuable information in the development of cancer prevention and treatment programs.

Proposed Course of Research:

Terminated

Publication:

Kramer, R.A and Andersen, N.: Isolation and characterization of low-phosphate induced genes of yeast. Proc. Nat. Acad. Sci. USA 77: 6541-6545, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05247-04 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of DNA-Dependent RNA Polymerase from Rooster Liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. P. Mullinix	Formerly Research Chemist (As of November 1979, Assistant Director for Intramural Planning)	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated.

Kastern, W.H., Christman, J.L., Eldridge, J.D., and Mullinix, K.P.: Estrogen regulates the number of RNA polymerase II molecules in rooster liver. Biochem. Biophys. Acta (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05251-03 LB

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure and Transcription of the Ribosomal Genes in Drosophila melanogaster

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	I. B. Dawid	Chief, Developmental Biochemistry Section	LB NCI
Other:	E. O. Long	Visiting Fellow	LB NCI
	R. K. Mandal	Visiting Scientist	LB NCI
	M. L. Rebbert	Chemist	LB NCI
	B. Wood	Lab. Worker	LB NCI
	W. Dimery	Biological Lab. Technician	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	1.6	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have determined the nucleotide sequences in several regions of the rRNA genes of Drosophila melanogaster. These include the region in which transcription terminates, and the boundaries between rRNA regions and insertions of type 1 and type 2. The termination region is of interest with respect to the fact that it does not show the string of T residues commonly found at this position. The boundaries of two type 1 insertions show target site duplications typical of transposable elements. In contrast, type 2 insertion boundaries do not show such duplications.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

Ribosomal RNA genes are of interest because they encode the most abundant RNA molecules in every cell. Drosophila rRNA genes further have the peculiar feature that some of the genes are continuous while others are interrupted by type 1 or type 2 insertions. We would like to understand the evolutionary origins and functional implications of ribosomal insertions in this organism. Further, we propose to study the functional requirements of rDNA expression. As a basis of studies in this direction we are analyzing the precise structure of critical regions in the rDNA molecule.

Methods Employed:

The sequencing method of Maxam and Gilbert was used throughout. For the determination of the termination site we employed a modification of the Berk and Sharp procedure, and direct RNA sequencing by enzymatic methods.

Schneider cells were cultured and nuclei were prepared by the detergent procedure. Such nuclei were incubated in vitro for the incorporation of labeled nucleotides into pre-initiated RNA chains.

Major Findings:

The 3' end of 28S rRNA and of 38S rRNA precursor was determined by S1 nuclease mapping and direct RNA sequencing, and was found to be identical. The nucleotide sequence of the rDNA around the 3' end of the RNA was determined. We found good homology of the 3' end of Drosophila rRNA with the rRNAs of yeast and Xenopus, but no homology of the spacer region immediately following the rRNA gene.

The nucleotide sequence of rDNA in the region of interruption of 28S rRNA was determined. Further, we sequenced one type 1 insertion of 1 kb and one insertion of 0.5 kb entirely. Both insertions show duplications at the target site of insertion, 14 bp for the 0.5 kb insertion and 11 bp for the 1 kb insertion. We sequenced three rRNA genes with type 2 insertions. These genes are interrupted about 70 bp upstream from the type 1 insertion. The point of interruption by type 2 insertions differed in the three cases studied. No target site duplication exists in type 2 insertions. Computer analysis has shown that the insertions do not have long terminal homologies in either the direct or inverted configurations.

Isolated nuclei from cultured cells of Drosophila were incubated in vitro under conditions where pre-initiated RNA chains are completed. This method allows the effective labeling of actively transcribed RNAs. In confirmation of earlier results in vivo we find that neither type 1 nor type 2 insertions are efficiently transcribed in Drosophila cells. In contrast, an easily detectable level of transcription was detected from the "nontranscribed" spacer. These results suggest that "read-through" through the termination site or initiation within the spacer, or both, occur fairly frequently in Drosophila Schneider cells.

Significance to Cancer Research:

The regulation of expression of interrupted genes is a basic question of cellular metabolism in all cells. Likewise, the structure and function of rRNA genes is of basic and general importance for normal and malignant cells. The suggestion that ribosomal insertions may have arisen by the introduction of transposable elements is also of significance for an understanding of the possible role of such elements in genomic rearrangements and the generation of new regulatory connections.

Proposed Course of Research:

We shall attempt the faithful transcription of rRNA genes in reconstructed systems, in particular in the frog oocyte. Once such systems are working they will allow a functional analysis of the effect of insertions on rRNA gene expression, and an analysis of the requirements for expression of uninterrupted genes.

Publications:

Long, E.O. and Dawid, I.B.: Repeated genes in eukaryotes. Annu. Rev. Biochem. 49: 727-764, 1980.

Long, E.O., Rebbert, M.L. and Dawid, I.B.: The nucleotide sequence of the initiation site for ribosomal RNA transcription in Drosophila melanogaster. Comparison of genes with and without insertions. Proc. Natl. Acad. Sci. USA 78: 1513-1517, 1981.

Long, E.O., Rebbert, M.L. and Dawid, I.B.: Structure and expression of ribosomal RNA genes of Drosophila melanogaster interrupted by type 2 insertion. Cold Spring Harbor Symp. Quant. Biol. 45, in press.

Mandal, R.K. and Dawid, I.B.: The nucleotide sequence of the transcription termination site of ribosomal RNA in Drosophila melanogaster. Nucleic Acids Res. 9: 1801-1811, 1981.

Long, E.O., Collins, M., Kiefer, B.I. and Dawid, I.B.: Expression of the ribosomal DNA insertions in bobbed mutants of Drosophila melanogaster. Molec. Gen. Genet., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05252-03 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Sequence Organization in the Genome of Drosophila melanogaster

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	I. B. Dawid	Chief, Developmental Biochemistry Section	LB NCI
Other:	E. O. Long	Visiting Fellow	LB NCI
	P. P. DiNocera	Visiting Associate	LB NCI
	M. E. Digan	Guest Worker	
	M. L. Rebbert	Chemist	LB NCI
	B. Wood	Lab. Worker	LB NCI
	W. Dimery	Biological Lab. Technician	LB NCI

COOPERATING UNITS (if any)

Dr. M.L. Pardue, Department of Biology, MIT, Cambridge, MA; Dr. M. Gans, CNRS, Gif-sur-Yvette, France; Dr. V. Pirrotta, EMBL, Heidelberg, West Germany

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.9	2.2	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The organization of repeated DNA elements interspersed with insertion-like sequences has been studied in D. melanogaster. These interspersed elements were shown to be homologous to sequences located on different chromosomal sites in different stocks of D. melanogaster, suggesting that the sequences are transposable in the genome. One such transposable element, named 101F, has been analyzed further. We have shown that it is a member of a divergent family of sequences, does not carry long terminal repeats, and that its insertion leads to a duplication of 13 base pairs at the target site. These properties define a new class of transposable sequences in Drosophila.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

Drosophila melanogaster is an excellent model for the study of gene arrangement and expression because of its small genome, and the accessibility of the organism to genetic and cytogenetic techniques. We are presently studying the arrangement and expression of a new class of transposable sequences in this organism. We have also begun to study a mutant that has functional relations with homeotic genes of the bithorax complex.

Methods Employed:

This project employs recombinant DNA methods to isolate different DNA molecules from D. melanogaster. We have prepared a set of clones which contain transposable elements. These elements are being used to determine the nucleotide sequence of selected regions by the Maxam/Gilbert procedure. We also use the cloned transposable elements to select additional homologous DNA molecules from a library of Drosophila DNA in lambda vectors.

In studying mutant flies we collaborate with M. Gans who carries out the genetic analyses. Methods employed in this project involve the analysis of labeled proteins on two-dimensional gels according to O'Farrell, and the use of a library of recombinant DNA molecules in a cosmid vector to be obtained from E. Meyerowitz at Cal. Tech.

Major Findings:

The sequences that flank or are interspersed with insertion-like elements in the chromocenter of D. melanogaster have been studied. Detailed analyses of chromosomal locations of such sequences in two stocks of D. melanogaster have revealed extensive site polymorphism between these stocks. This finding suggests that the sequences studied are transposable in the genome. One such sequence, named 101F, interrupts an insertion-like element; the nucleotide sequence of the boundary regions has been determined and compared with the sequence of the ribosomal insertion itself. The boundaries show a target site duplication of a stretch of 13 base pairs that occur only once in the ribosomal insertion itself. Such target site duplications are typical of transposable elements. Within the 101F element itself we did not find any long repeated sequence, neither in the direct or inverted configuration.

The mutant fs(1)h has been detected and characterized by M. Gans and her colleagues. It can lead to homeotic transformations in the fly and also carries a temperature-sensitive female sterile phenotype. Through collaboration with V. Pirrotta we have obtained a set of cloned DNA molecules that are derived from the chromosomal region of the fs(1)h mutation. These cloned molecules are being used to collect a larger set of molecules that will cover the entire region around fs(1)h.

Significance to Cancer Research:

Inasmuch as cancer is thought to involve a failure of basic cellular regulatory processes we need to understand in much more detail the structural basis and molecular mechanism of these processes. The organization and expression of the genome in animal cells is a subject of central importance in the analysis of regulatory processes during growth, development and evolution. As mentioned above Drosophila melanogaster is a particularly suited organism for the study of genome organization and function. The presence of transposable elements in the animal genome is an important recent finding. These elements may have great significance in the evolution of animal genomes and in changes that may alter major regulatory pathways.

Proposed Course of Research:

Sequences homologous to the transposable element 101F will be isolated and their properties will be studied. We shall analyze the nature of additional transposable DNA elements in Drosophila. The gene which is mutated in fs(1)h will be studied by an attempt to isolate the DNA carrying this gene by molecular cloning.

Publication:

Pardue, M.L. and Dawid, I.B.: Chromosomal locations of two DNA segments that flank ribosomal insertion-like sequences in Drosophila: Flanking sequences are mobile elements. Chromosoma, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05253-03 LB

PERIOD COVERED: October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Sequence and Expression of the Vitellogenin Genes in Xenopus laevis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	I. B. Dawid	Chief, Developmental Biochemistry Section	LB NCI
Other:	B. Wood	Lab. Worker	LB NCI

COOPERATING UNITS (if any)

G.U. Ryffel, Assistant Professor, R. Weber, Professor, Department of Zoology, University of Bern, Switzerland; W. Wahli, University of Lausanne, Switzerland

LAB/BRANCH: Laboratory of Biochemistry

SECTION: Developmental Biochemistry Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.1	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In Xenopus laevis, vitellogenin which is synthesiged in the liver under control of estrogen is encoded in a small family of genes. Two of the vitellogenin genes together with long stretches of their flanking regions have been isolated from a Xenopus gene library. The structural organization of these two genes has been determined by electron microscopy. In both genes, the mRNA coding sequence is interrupted 33 times by sequences (introns) not present in mature vitellogenin mRNA. We have determined the distribution of repeated DNA sequences in and around the two vitellogenin A genes. Many of the introns contain sequences that are repeated elsewhere in the genome. We suggest that intron evolution may involve the insertion and deletion of mobile repeated DNA elements.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

Isolation of the genomic sequences containing the vitellogenin genes and characterization of the structural organization of the genes and of the gene family.

Methods Employed:

Cloned DNA molecules from a library of Xenopus DNA fragments had been obtained earlier. In the present study the distribution of repeated sequences was determined by DNA blotting according to Southern, and by electron microscopy of heteroduplex molecules.

Major Findings:

Both the A1 and the A2 vitellogenin genes contain many regions that are repeated elsewhere in the genome. Since the coding regions (exons) are present in only two related copies (A1 and A2) we conclude that many introns contain repeated DNA. The introns of the A1 gene are not homologous to the introns of the A2 gene; therefore, the two vitellogenin genes contain repeat elements belonging to different families at analogous positions. The A1 gene is considerably (by about 5 kb) longer than the A2 gene, and it also contains more repeated DNA.

Gene A1 occurs in two polymorphic forms, a short form and a long form in which intron 11 is longer by about 350 bp. In the long but not in the short form of gene A1 intron 11 contains a repeated DNA sequence. We suggest that the polymorphism arises by the insertion (or deletion) of a repeat element into (or from) intron 11.

In gene A2 we observed a repeated element in an intron that is homologous to a DNA stretch in the 5' flanking region of the gene. The two homologous elements occur in inverted configuration. Thus, repeat elements can be found in both orientations with respect to coding regions.

On the basis of these observations we suggest the hypothesis that some repeated sequences in Xenopus may be transposable, and that they may be inserted into and deleted from introns as well as flanking regions of the vitellogenin genes (and presumably other genes as well). This suggestion accounts for the rapid divergence of intron sequence and length that we observe in the vitellogenin gene family.

Significance to Cancer Research:

In Xenopus, the small family of vitellogenin genes is expressed under the control of estrogen. Forms of cancer are probably the result of the action of steroids on gene expression or of the loss of hormonal control on gene activity. Vitellogenesis, by its features, is a good model system to investigate mechanisms of hormonal regulated gene expression whose malfunction may produce cancer as indicated above.

Proposed Course of Research:

Terminated.

Publication:

Wahli, W., Dawid, I.B., Ryffel, G.U. and Weber, R.: Vitellogenesis and the vitellogenin gene family. Science 212: 298-304, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05254-03 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Gene Expression in Xenopus laevis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	I. B. Dawid	Chief, Developmental Biochemistry Section	LB NCI
Other:	B. K. Kay	Guest Worker	LB NCI
	Y. H. Chien	Expert	LB NCI
	T. Sargent	Staff Fellow	LB NCI
	B. Wood	Lab. Worker	LB NCI
	W. Dimery	Biological Lab. Technician	LB NCI

COOPERATING UNITS (if any)

M.B. Dworkin and J.W.B. Hershey, Department of Biological Chemistry, University of California Medical School, Davis, CA

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.6	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are analyzing the expression of genes active during early embryo development in the frog Xenopus laevis with cloned cDNAs. In a library of 200 cloned sequences that code for abundant RNAs in two embryo stages, we have identified transcripts of some structural and some mitochondrial genes. A significant proportion of cDNAs in this library contain sequences that are moderately repeated in the genome. To analyze stage-specific RNA species we have started to construct a DNA library of particular RNAs which are present in gastrula embryos but absent in oocytes. To analyze the developmental expression of defined genes, we are constructing cDNA clones for two calcium binding proteins, calmodulin and parvalbumin.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

The analysis of developmental expression of a large set of genes that are active in embryogenesis is the goal of this project. The structure and functional regulation of a number of genes, and the cellular distribution and function of their products will be studied.

Methods Employed:

In addition to preparation and screening clone libraries we have been employing DNA sequence determination, RNA ("Northern") and DNA ("Southern") blot techniques, and hybrid selected mRNA translation in vitro.

Major Findings:

A set of 240 clones containing cDNA sequences from stage 10 and 41 X. laevis embryos were analyzed by colony hybridization. With heterologous chicken cDNAs as probes we have detected α-actin and β-tubulin sequences, but no α-tubulin, β-actin, or vimentin sequences. We have also tested this library for mitochondrial sequences and found them to be prevalent. Mitochondrial sequences were found to be highly abundant in gastrula stage poly(A)+RNA sequences; in tadpole RNA their relative abundance is reduced several fold. Mitochondrial sequences account for the most abundant poly(A)+RNA molecules in the gastrula population. The high abundance of mitochondrial RNA in early stages may be a consequence of the accumulation of large numbers of mitochondria in the egg. We have tested this cDNA library for sequences that are repeated in the frog genome and found that approximately 10% of the cloned cDNAs contain sequences present in a thousand or more copies. Southern blot experiments show that the repeated elements are interspersed with other DNA, with one exception, where the repeats are tandemly arranged. Northern blot experiments show that each cDNA derived from repeated genes corresponds to a small number of discrete-sized RNAs in embryos.

We have started to analyze gastrula-specific RNAs by isolating gastrula cDNA that fails to anneal with an excess of ovary poly(A)+RNA. About 1% of the gastrula RNA appears to be at least 20 times more abundant in gastrulae than in oocytes. To study this class of RNA which may be important in directing gastrulation, cDNA clones will be prepared from the unreacted cDNA that has been purified by hydroxylapatite chromatography.

We propose to compare the developmental behavior of an ubiquitous gene (calmodulin) with that of a highly specialized gene (parvalbumin) during embryogenesis and morphogenesis in Xenopus laevis. Both proteins bind calcium, and amino acid sequence comparisons indicate that they are derived from a common ancestor. Calmodulin is found in all eukaryotic cell types and its amino acid sequence has been conserved through evolution. The calmodulin-Ca^{++} complex mediates a variety of cellular processes through interaction with other proteins, and the regulation function is neither tissue-specific nor species specific. In comparison to calmodulin, parvalbumin has lost its ability to interact with other proteins, but it can bind calcium and serve as relaxing factor in fast relaxing muscle and is found only in this type of tissue. Another interesting contrast with calmodulin is that it is highly species specific.

We are currently constructing cDNA clones of calmodulin and parvalbumin. We are making cDNA clones using size-fractionated RNAs which have been enriched for the sequences of interest. We then identify the cDNA clones by hybrid-select translation followed by antibody precipitation and nucleotide sequencing. The cDNA clones will then be used as probes for the study of gene regulation in different stages of Xenopus laevis development.

Significance to Cancer Research:

The aim of the project is a better understanding of developmental regulation of gene activity. More information on this subject will be important for an understanding of the biological basis of cancer.

Proposed Course of Research:

Various genes that exhibit interesting properties such as novel mRNA structure, tissue-specific expression or rapid induction early in development will be identified and isolated from libraries of cloned mRNA and genomic sequences. Analysis of the function and expression of this small collection of genes should help to illuminate the involvement of the genome in the control of early development.

Publication:

Dworkin, M.B., Kay, B.K., Hershey, J.W.B. and Dawid, I.B.: Mitochondrial RNAs are abundant in the poly(A)$^+$ RNA population of early frog embryos. Develop. Biol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05256-03 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of Expression of Bacterial Nitrogen Fixation Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. R. Appelbaum	Staff Fellow	LB NCI
Other:	R. A. Kramer	Senior Staff Fellow	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The genes which control nitrogen fixation in Klebsiella pneumoniae strain M5a1 have been isolated and analyzed using recombinant DNA techniques. The isolated genes have been used to study the arrangement and expression of nif genes in M5a1 and other Klebsiella strains.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

The goal of this research is to elucidate the molecular mechanisms involved in regulation of the expression of the genes involved in nitrogen fixation in bacteria. This process is thought to involve many genes (nif genes) which are coordinately controlled by a number of different physiological parameters such as ammonia concentration, molybdenum concentration, oxygen partial pressure, glutamine synthetase, cyclic nucleotides, and interaction of nitrogen-fixing bacteria with eukaryotic plant cells. This project is designed to reveal novel mechanisms of genetic regulation.

Methods Employed:

Recombinant DNA techniques, restriction enzyme analysis, in vitro transcription, and nucleic acid hybridization techniques are being used to map nif genes and their RNA transcripts on Klebsiella DNA, and to compare Klebsiella pneumoniae strain M5a1 nif genes with genes in other strains.

Major Findings:

Segments of K. pneumoniae M5a1 chromosomal DNA which collectively contain all of the nif genes have been cloned in E. coli using the plasmid pBR322 as a vector. These recombinant plasmids provide an abundant source of nif DNA for detailed molecular studies. Many different restriction enzymes have been used to analyze the cloned segments and thereby construct a detailed physical map of the cloned nif region. The cloned segments have been used in hybridization experiments with DNA prepared from other Klebsiella strains found in clinical and environmental settings. Our results indicate that the structure of the nif region is very similar in Nif^+ strains from different sources, whereas Nif^- K. pneumoniae strains contain little or no homologous DNA. We have also shown that the cloned genes can be used to identify mRNA molecules transcribed in vivo from nif genes in strain M5a1. These studies have shown that at least some of the nif operons are regulated at the level of transcription. One unexpected finding was the discovery of two different sized mRNA molecules that are produced from a single nif operon.

To identify the DNA sequences which control expression of nif operons, we ligated small fragments of nif DNA to the E. coli gal kinase structural gene, and then introduced the recombinant molecules into E. coli strains in which we could measure expression of the kinase gene. Several putative nif promoter fragments were then mapped on the Klebsiella chromosome. In vitro transcription was used to map the transcription start site in one such fragment in anticipation of DNA sequencing of the promotor.

Significance to Cancer Research:

An understanding of the molecular mechanisms involved in gene expression will be necessary to understand the processes by which a cell loses its genetic regulation and becomes cancerous. This project is designed to determine part of the normal mechanisms of gene expression and regulation as a basis for identifying the changes which have occurred at the molecular level in malignant cells.

Knowledge of these changes should provide valuable information in the development of cancer prevention and treatment progams. The principal organism used in these studies, Klebsiella pneumoniae, is a human pathogen which frequently causes life-threatening infections in debilitated individuals, including some cancer patients. This project will provide information about the genetic regulation of a major metabolic pathway which occurs in Klebsiella pneumoniae under anaerobic conditions, and will thereby contribute to our understanding of the pathogenicity of this organism.

Proposed Course of Research:

Terminated.

Publication:

Appelbaum, E.R. and Kramer, R.A.: Restriction mapping of deletions in the nif region of the Klebsiella pneumoniae chromosome. Molec. Gen. Genetics 179: 349-354, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05257-03 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Transcription of SV40 DNA-Protein Complexes by DNA-dependent RNA Polymerase II

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: K. P. Mullinix — Formerly Research Chemist (As of November 1979, Assistant Director for Intramural Planning) — LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05258-02 LB

PERIOD COVERED: October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Molecular Studies of Eukaryotic Gene Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. M. Paterson	Research Chemist	LB NCI
Other:	Z. Zehner	Guest Worker	LB NCI
	M. C. O'Neill	API	Dept. Biol. UMBC
	J. Eldridge	Technician	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH: Laboratory of Biochemistry

SECTION: Developmental Biochemistry Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The proteins encoded in the most abundant mRNA class expressed in embryonic chick muscle have been identified and include the following: Myosin heavy chains (M-HC), myosin light chains (M-LC), α and β actin, tropomyosin, vimentin, desmin, glyceraldehyde phosphate 3' dehydrogenase (GAP), muscle and brain creatin phosphokinase (CPK). Certain isozymic forms of these proteins are expressed only in differentiated muscle e.g., myosin heavy and light chains, α actin, and muscle CPK whereas all cells express the other forms of these proteins. In addition, the muscle specific set of proteins appears to be coordinately regulated in that all these genes are switched on synchronously during differentiation. Using defined, cloned ds cDNA probes for various of these genes we have isolated genomic sequences for, α and β actin, vimentin, myosin heavy chain and GAP. We are comparing the organization, structure, and regulation of the set of differentiation specific and the constitutive genes. At present we are focusing on α and β actin and vimentin.

PHS-6040
(Rev. 2-81)

Project Description:

(1) To prepare ds cDNA probes for the proteins of interest, and to utilize these probes to isolate the genomic sequences for structural studies. These studies primarily involve electron microscopic analysis and sequence analysis of all or part of the transcriptional unit for a given gene.

(2) To define the transcriptional start sites, splice junctions and termination signals for the genes of interest. This information will be used in a comparative study of differentiation specific and "house keeping" sequences.

(3) Some of the ds cDNA probes are to be used in chromosomal localization studies on mouse chromosomes. Several of the structural genes under investigation are highly conserved across species. It is of interest to know if isozymes are proximal or distal on the same chromosome, or on different chromosomes.

(4) When the promotor regions for various genes have been identified, it is intended to analyze promotor function in one of the eukaryotic vector systems now available. We intend to: 1) see if a promotor for a differentiation specific gene, such as α actin, can function when placed in an undifferentiated cell or if differentiation is required for function; 2) define the essential sequence elements for promotor function; 3) determine the role of the intron arrangement in the regulation of gene expression.

Methods Employed

Specific ds cDNA probes are being prepared from A^+ mRNA fractionated on preparative methylmercury gels. Fractions enriched for a particular mRNA, as judged by analysis in a cell-free protein synthesizing system, are cloned using standard methods. Clones are identified by positive selection.

The probes are used to screen the charon 4A library of the chicken genome with standard procedures, or with the supressor miniplasmid method of Maniatis. The distribution of the coding information and its polarity within a given isolate is determined by hybridization with kinased RNA and short cDNA. The 5' proximal and 3' proximal fragments in the appropriate restriction digest are sequenced to clearly determine the end points of the transcriptional unit for each gene. Electron microscopic studies give the information on the intron-exon pattern and the relatedness of the various isolates for a given gene. Restriction fragments containing the promoter regions will be subcloned into one of the eukaryotic vector systems for further analysis.

Major Findings:

The actin gene family: Using an α actin ds cDNA probe of roughly 1 Kb, 13 different actin isolates were prepared from the charon 4A chicken genome library. The large α actin probe will select α, β, γ actins since the probe represents coding regions homologous in all the actins. This group was screened further with α and β specific probes. Clones containing all the hybridized information in a single R_1 fragment for either α or β actin were selected and the appropriate R_1 fragment subcloned in pBR 325. The vector contains an R_1 site in a chloramphenicol resistant gene, allowing one to subclone R_1 fragments

with a positive selection. In the α group, restriction analysis with Hind III, Bgl II, Sma I, and Kpn I revealed three overlapping α clones, and one unique α clone. Only one β clone was selected from the initial 13 isolates. Hybridization studies with kinased RNA and short cDNA have allowed us to determine the distribution and polarity of the coding information in the clones.

The 5' proximal restriction fragments have been isolated and sequence studies are underway. Electron microscopic analysis reveals at least 2 introns in the overlapping α gene. The others are under study. Preliminary copy number experiments suggest 1-2 copies of the α and β gene per haploid genome in the chicken.

Vimentin gene family: Genomic clones for the intermediate filament protein, vimentin, have been isolated and identified by mRNA selection, translation, and product analysis by both isoelectric focusing and immuno-precipitation. From heteroduplex analysis 15 independent isolates were shown to contain overlapping coding information for the same gene. Copy number experiments indicate that the vimentin gene is present in the chick genome (haploid) in only one to two copies. Hybridization to DNA digested with various restriction enzymes (Southern analysis) yielded hybridization to expected fragments as deduced from restriction maps of the vimentin clones. From these results it would seem that vimentin is a single copy gene. However, analysis of either total or poly A^+ mRNA extracted from muscle and sized on methyl mercury gels (Northern analysis), yields two distinct mRNAs differing in molecular weight by approximately 300 nucleotides. These two mRNA's are equally represented in muscle at various stages in muscle development isolated from either chick embryos or myogenic cultures. This result could be due to any combination of the following reasons, i.e., a heterogeneity in the 3'- or 5'-end of the vimentin mRNA, a splicing difference, a precursor-product relationship, or cross-hybridization to the nucleotide sequence of a closely related intermediate filament protein such as desmin. It is felt that this possibility is unlikely since skeletal muscle at some of the stages utilized is synthesizing little or no desmin by either protein analysis or mRNA translation. However, this possibility can be further excluded by RNA selection with mRNA extracted from a tissue known to contain desmin, i.e., chicken gizzard. Likewise, a precursor-product relationship is unlikely since the two mRNA species are always present at equal, fairly abundant amounts. The other possibilities are being tested by analysis of vimentin cDNA clones (8 have been isolated) by restriction mapping, heteroduplexing, and by S1 mapping of the genomic clones. The S1 experiments will also be useful in delineating intron exon patterns and localization of the 5'- end of the gene in conjunction with other experiments of interest within our research group. Preliminary experiments do show that the vimentin mRNA heterogeneity does not reside at the 3'- end since equal hybridization is obtained with a short (200 nts) versus a long (800 nts) 3'-end cDNA probe.

Future experiments entail sequencing parts of these clones in conjunction with Dr. Peter Steinert (Bldg. 10) to be used in deducing the amino acid sequence of vimentin, a protein difficult to sequence due to its low abundancy and a blocked amino-terminus. Likewise, we plan to utilize the various cDNA probes isolated in the lab (actins, vimentin, GAP, myosin heavy chain) for analyzing mRNA abundancy following viral transformation. At least one of these proteins (vimentin) has been shown to be specifically phosphorylated by RSV SrG^{60}.

Projected Course of Research:

We intend to analyze the organization, structure and regulation of constitutive and differentiation specific genes.

Publication:

Paterson, B.M. and Roberts, B.E.: Structural gene identification utilizing eukaryotic cell-free translational systems. Gene Amplification and Analysis, Vol. II (J.G. Chirikjian and T.S. Papas, eds.) 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05259-02 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Role of mRNA Structure and its Role in the Regulation of Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.P. Paterson	Research Chemist	LB NCI
Others:	M. Rosenberg	Research Chemist	LB NCI
	C.L. Queen	Staff Fellow	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Prokaryotic messenger RNAs of known sequence of the 5' termini have been used to study the role of mRNA structure in protein translation. Structural features within the mRNA which regulate the rates of ribosome binding and AUG site selection have been determined utilizing these defined sequences in both prokaryotic and eukaryotic cell free protein translational systems. Dr. Queen is preparing the manuscript of this study.

Project Description:

Objectives:

1. Prepare a set of mRNAs of defined sequence and structure for functional analysis in protein translational systems.

2. Determine those structural features in the mRNA which regulate mRNA function, i.e. rates of ribosome binding, AUG site selection, reutilization frequency, 1/2 life of mRNA.

Methods:

1. Transcribe prokaryotic genes *in vitro* with *E. coli* polymerase II to obtain defined mRNA transcripts.

2. Modify mRNA structure utilizing recombinant DNA technology to create new transcriptional units for a given gene.

3. Modify mRNA transcripts with the enzymatic addition of various functional groups, i.e. cap moieties or methyl groups.

4. Analysis of mRNAs in prokaryotic and eukaryotic translational systems: rates of translation, ribosome binding, and position of ribosome binding sites utilizing protection-sequencing experiment.

Major Findings:

Eukaryotic ribosomes recognize the same ribosome binding sites as prokaryotic ribosomes both on mono- and polycistronic mRNAs. However, eukaryotic ribosomes will not initiate translation on binding sites internal to the 5' proximal binding site whereas prokaryotic ribosomes will. The proximity to the cap structure appears important in the case of eukaryotic ribosomes.

Projected Course of Research:

To continue to examine mRNA structure and its role in the regulation of gene expression.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05260-02 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Repeated DNA Sequences in the Chicken Genome: Structure and Methylation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	Francine C. Eden	Staff Fellow	LB	NCI*
OTHER:	D.A. Sobieski	Chemist	LB	NCI
	Michael Lerman	Visiting Scientist	LB	NCI

*Present address LMC, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In the chicken genome there are large regions occupied not by structural genes but by repetitive DNA sequences in which many of the cytosine residues have been methylated to 5-methyl cytosine. We are determining the detailed structure and function of these methylated repetitive regions. We study their organization by molecular cloning, by Southern transfer hybridization experiments, and by in situ hybridization to metaphase chromosomes. We have determined their pattern of methylation in sperm, embryonic stages, adult tissues, contemporary populations, and in avian species related to chickens. Methylation is maintained in all DNA samples mentioned above. By direct DNA sequencing we have analyzed the nucleotide sequences that surround these methylated bases. These DNA sequences have also been used in analysis of DNA binding proteins isolated from nuclei. Completion of this work will provide a full description of the structure of some persistently methylated repeated DNA sequences and the influence of methylation on DNA-protein interactions.

PHS-6040
(Rev. 2-81)

Project Description:

Introduction and Objectives

Many different kinds of DNA sequences are present in the eucaryotic genome, but it is not yet possible to assign to each of them a distinct cellular function. Among the most intriguing regions are those occupied entirely by repetitive DNA sequences. In the chicken genome, there are many such regions whose internal organization has been determined by previous work in this project. The repeated sequences occur in clusters greater than 20 KB in length and the component sequence elements are in a different order in each cluster. We also know that these particular genomic regions contain a high content of modified DNA bases (cytosine modified to 5-methyl cytosine). Thus they are a distinct structural entity whose function needs to be determined. One specific aim of present work is to understand the structure of these large methylated regions in more detail.

Another aspect of this study is to test the interaction of methylated DNA sequences with DNA binding proteins from the cell nucleus. We are looking either for direct binding of certain proteins to specific DNA sequences or for non-specific interactions of the type that could influence the stability of the DNA helix or chromatin conformation.

Methods Employed

We use cloned DNA fragments that correspond to repeated DNA sequences having a clustered organization in the chicken genome. For structural analysis of these clusters, we use 20 KB segments cloned in phage lambda. In other aspects of the work, smaller segments cloned in the plasmid PBR322 are used. The modes of analysis include Southern transfer hybridization experiments using total chicken DNA, restriction mapping of cloned DNA segments, analysis of heteroduplex structures by electron microscopy, DNA sequencing using the procedure of Maxim and Gilbert, and in situ hybridization to metaphase chromosomes.

For the characterization of DNA binding proteins, nuclei are prepared from chicken liver or sperm by the Cheuveau procedure. Nuclear proteins are freed of endogenous DNA by digestion with DNAse I and are fractionated either into acid soluble and acid-insoluble fractions or into fractions based on salt solubility. The proteins are then separated by electrophoresis through SDS polyacrylamide gels and are transferred to nitrocellulose sheets by the method of Weintraub and Laemmli (Western blots) for subsequent analysis. The cloned probes described above are labelled with ^{32}P and "hybridized" to the protein blots. We look for interactions that are stable in high salt or for those that result in protection of the DNA probe from digestion by DNAse I. The specificity of the interaction is tested by competition with heterologous or homologous DNA.

Major Findings

1. Length and Distribution of Repeated DNA Clusters in the Chicken Genome.

In the initial series of experiments, it was determined that when clusters of repeated sequences are selected from the library of chicken DNA cloned in phage lambda, they occupy the entire inserted DNA segment in each phage. This indicates that the clusters usually exceed 20 KB in length. To further investigate their dimensions, we selected additional phages using probes designed to select for boundaries between repeated sequence clusters and other DNA sequences. Of fifteen recombinants selected, none contained such boundaries, from which we conclude that the clusters usually exceed 50 KB in length. They constitute some of the largest repetitive regions described in any eucaryotic genome so far, except for those with a tandemly repeating organization clearly different from that described here. We turned to cytological methods to ascertain how many different locations in the genome bear these repetitive clusters. Colcemid arrest was used to develop many metaphase chromosome displays in chicken cells growing in tissue culture. Repeated sequence probes labelled to high specific radioactivity were hybridized to the fixed chromosomes denatured in situ. The autoradiographic results show hybridization to many different chromosomes, with some examples of two detectable loci on a single chromosome. Thus these repeated DNA clusters are at least somewhat dispersed in the genome. These are certainly not restricted to centromeric regions. Models of function that we formulate and test will now take into account two facts: repetitive DNA clusters are very large and they occur on many different chromosomes in the chicken karyotype.

2. Methylation of Repeated DNA Clusters

Methylation within repeated DNA clusters has been extensively analyzed using restriction endonucleases sensitive to methylation. We found a pattern of methylated and unmethylated sites that is reproduced among most, if not all, of the copies of these sequences in the genome, which numbers about 500. Also, it is clear that a significant amount of methylation affects all of the cluster elements, indicating that the methylated regions are as large as the clusters themselves.

These collected facts contrast with literature reports on methylation in and around structural genes, where it is clear that methylation is not so extensive and that its presence is negatively correlated with the expression of genes, that is, it varies with cell type. These differences led us to investigate whether the methylated regions described above are modified to the same extent in different cell types and whether their methylation is maintained during embryonic development. We found that in repeated DNA clusters the methylation is very persistent and does not seem to vary in dozens of different DNA sources. We included DNA from sperm, from whole embryos of various stages, from individual embryonic tissues, from adult tissues, from 30 different individual chickens, and from five avian species related to chickens. These large repetitive regions are apparently constitutively methylated in the chicken genome and are even methylated in related species. This implies that methylation directed toward this component has a different role than the variable methylation found near structural genes.

We found the DNA of sperm to be even more extensively methylated than that of somatic cells. This one fact suggests to us that the large methylated regions could play a role in chromatin condensation, where sperm represent the extreme of packing requirements. This point gains some support from the protein.

3. Nucleotide Sequence of Methylated Regions

It has now become a generality that methylation of DNA is introduced after DNA has been polymerized. We presume that there are specific methylases that recognize certain DNA regions and modify cytosine residues there. It is important to understand what structural features determine whether methylation will occur. We have examined cloned DNA segments that correspond to methylated regions in the chicken genome by direct DNA sequencing. So far we have sequences around about ten methylated sites, and when sufficient data has been accumulated, computer analysis will be used to look for common sequences. With the limited data collected so far, it seems clear that methylated regions are high G+C, and contain a high proportion of homopolymer tracts. Often the methylated site is preceded to the 5' side by a long tract of C's and T's. Additional data will determine whether these correlations hold. The ultimate goal of this aspect of the project is to identify a specific nucleotide sequence that could be used to identify methylating enzymes according to their specificity. This would be an important step in understanding the control of methylation in the genome.

4. Characterization of DNA Binding Proteins

Dr. Lerman has succeeded in obtaining displays of nuclear proteins bound to nitrocellulose with sufficient separation to identify about 40 bands that interact strongly with the repeated DNA probes. There are obvious differences between DNA binding proteins in sperm and in somatic nuclei. In the molecular weight range 6-10 KD, he detects 4-5 proteins that comprise about 50% of the protein mass in sperm and only 1-2% in liver nuclei. In proteins for somatic cells there is extensive binding to cellular histones in the 12-30 KD range. Other, non-histone proteins of similar molecular weights are also readily detected; probably they belong to the "high mobility" group of non-histone proteins described by others. More than ten high molecular weight proteins are also detected. Some of these may be part of the nuclear "matrix".

One of the repeated sequence probes seems to have a very high affinity for a particluar protein of 75 KD size. This particular complex is stable in salt up to 1M NaCl, conditions which result in almost complete dissociation of histone-DNA complexes. It binds the probe in the presence of a 10^4-fold excess of heterologous DNA and it protects a substantial fraction of the bound DNA from digestion by DNAse I. Preliminary data indicate that this 75 KD protein is part of the nuclear matrix.

These results suggest to us that the large methylated regions play a role in the organization of chromatin and its state of condensation. These functions are probably mediated through specific DNA-protein interactions and may be influenced by methylation.

Significance to Cancer Research

Our work is part of a broad effort to relate the structural organization of eucaryotic DNA to cellular functions. The success of this endeavor will require both structural information about the kinds of DNA sequences present in the genome and the design of experiments which reveal the functions of each kind of sequence. This information will be the basis of understanding the control of normal growth and differentiation. The parallel understanding of malignant cell function will surely contribute to understanding cancer.

Proposed Course of Research

We propose to bring our understanding of the structure of clustered repeated DNA sequences to a new level this year. So far we have investigated their organization on a general level but now more detailed comparisons are required. One important question is how similar are the different copies of these sequences. Apparently most of them are methylated and most of them interact with certain DNA binding proteins, so the structural requirements for this can be deduced by determining to what extent different copies are similar. We will focus on a single cluster sequence element 3.6 KB in length. We have already selected 15 different copies of this sequence from the library of chicken DNA cloned in lambda. These will be sub-cloned into pBR322 and compared by heteroduplex analysis, by S1 nuclease digestion experiments, and by blot hybridization using the "Southern cross" format. We also intend to sequence corresponding regions among the different segments to determine whether regions surrounding methylated bases are identical in sequence. Lastly, if structural variants are found, they will be used individually in protein binding experiments to determine the effect of sequence variability on binding activity.

With respect to the DNA binding proteins, we wish to pursue the 75 KD binding protein that is probably part of the nuclear matrix. This will include (1) isolation and purification of this protein; (2) investigation of the sequence specificity of binding by Me_2SO_4 protection and DNA footprinting; and (3) tests for enrichment of the repeated sequence which binds this protein in matrix-associated DNA. Also new DNA-binding proteins will be sought using related repeated DNA probes.

Publications

Eden, F.C., Musti, A.M., and Sobieski, D.A.: Clusters of chicken repeated DNA sequences are extensively methylated but contain specific unmethylated regions. J. Mol. Biol., in press.

Musti, A.M., Sobieski, D.A., Chen, B.B., and Eden, F.C.: Repeated DNA clusters in the chicken genome contain homologous sequence elements in scrambled order. Biochemistry, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05261-02 LB

PERIOD COVERED

September 30, 1980 to October 1, 1981

TITLE OF PROJECT (80 characters or less)

Cellular Manifestations of Cancer in Human Carcinomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Cecil Fox — Senior Scientist — LB NCI

COOPERATING UNITS (if any) Department of Biochemistry, Lunds Universitet, Lund, Sweden; Departments of Surgery and Oncology, Walter Reed Army Medical Center; Department of Pathology, University of Maryland; SSDZ Department of Anatomical Pathology, Delft, Holland.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this program are to use biological characteristics of tumor cells as a means for improving grading of human cancer through use of more objective parameters. The object of grading tumors is to estimate the potential of the tumor for growth, invasion, and metastasis, which are important factors to clinicians in planning therapy. Tumor cell attachment to new substrates is being compared to normal cell attachment using reflection contrast microscopy. A major characteristic of cancer cells, release from density dependent growth control, is also being studied as is the role of lamellar cytoplasm in tumor cell growth. The distribution of enzyme sites on the membrane surface of human oncogenic fibroblasts has been compared to the distribution in nononcogenic cells. Some new projects characterize human cancer cell populations in archival tissues and with needle aspiration cytology. A study of endometrial epithelium and its growth dynamics in relation to endometrial hyperplasia is now underway, and a separate project on the dynamics of cellular response to cytostatic agents in head and neck cancer is in the process of activation.

PHS-6040
(Rev. 2-81)

Project Description

Objective: The grading of cancers is an estimate of the biological character of a tumor cell population and is usually done by subjective appraisal. This project is designed to provide the pathologist and the clinician with objective signs and data bases that may be included in the information used in deciding the course of treatment of human cancers. The project is a broad one that seeks to identify characteristics of human carcinomas that may be used for interpretation of the biological state of cancerous or precancerous cells.

Methods Employed: The methods employed will be described under each study heading.

Study 1. Cell attachment mechanisms and the formation of cell contacts with substrates.

The attachment of cells to substrates may be involved in the process of metastasis and could perhaps be used for characterizing cellular populations of cancers. The mechanics of cell attachment to glass surfaces using reflection contrast microscopy continues and as more human cells become available, time-lapse cinephotomicrographs will be made of the process so that the dynamics of this differentiation process in epithelial cells may be identified.

Study 2. Cytological characterization of head and neck tumors.

A project in collaboration with the Department of Oncology, WRAMC, involves following a group of patients with both operable and inoperable cancer using needle aspiration cytology for repeated cell sampling. The cells so obtained will be characterized as to their labeling index, amounts of nuclear DNA, cell and nuclear size. These values will be determined on a series of samples taken as the patients are treated by chemotherapy. We believe that we can obtain some interesting observations about which cells are affected by chemotherapy and especially about the meaning of clonogenic models for a proliferating stem cell in head and neck carcinomas.

Study 3. Metaplasia in uterine epithelium.

The process of metaplasia in epithelial surfaces is often believed to be a first step in the carcinogenic process. It is possible to introduce metaplasia in vitro. This study concerns the cellular events of metaplasia as determined by light and electron microscopy and to try to establish which cell populations in the endometrium proliferate to metaplasia.

Study 4. Quantitative parameters useful in differentiating endometrial hyperplasia from endometrial carcinoma and CIS.

Exploratory studies have shown hyperplasia from endometrial carcinoma and CIS. Exploratory studies have shown that in endometrial tissues that subjectively are of similar structure and which could either be endometrial hyperplasia or endometrial carcinoma, known cases of endometrial carcinoma will have glandular cells that are aneuploid while those from hyperplasias will rarely exceed 4n amounts of DNA. This added factor may be of use to pathologists in decision-

making and while not diagnostic, provides one more piece of information in the logic of diagnosis. Amounts of DNA are determined by microfluorimetry using an improved microfluorometer. With known archival specimens it will be possible to investigate other cellular parameters such as nuclear density of stromal cells, or of geometric relationships between cell types.

Study 5. Distribution of enzyme loci in non-neoplastic and oncogenic human fibroblasts.

The most significant difference between oncogenic and non-neoplastic fibroblasts is their ability to form lamellar cytoplasm when fully spread on a substrate. This means that membrane mounted enzymes could be present in greater or lesser amounts in transformed cells. Early studies indicate that actually there are the same quantities of some enzymes in both types. This indicates that the density or proximity to each other of the proteins may be much greater in transformed cells. Consequently, membrane transport may be considerably different after transformation. So far, this study has used NADH ferricyanide reductase for this study, but for technical reasons some of the membrane bound phosphorylases may be easier to study.

Study 6. The role of lamellar cytoplasm in neoplastic transformation.

A study continues on lamellar cytoplasm in growth regulation of cells. Cells growing on surfaces continue to grow when they can spread their cytoplasm maximally. When cell crowding becomes great enough, cells cease to divide and may go over to the G_0 phase of the cell cycle. While not entirely similar to human epithelium, rabbit renal epithelium may be dissected in the form of renal tubules and specific portion of the tubule cultured on collagen membranes. We are currently studying the growth of such monolayers in relation to development of renal function by the epithelial cells.

Significance:

Cancer in humans may be part of the human estate: that is, those events that lead to cancer might also lead to the irreversible differentiation of the cells of human epithelium. If this is true, that cancer is the result of some number of transpositions or even erradications in the genome, then the most immediate benefit to the cancer patient would be research to improve his chances with existing treatment modalities. This program is intended to investigate such a possibility and further, to investigate some of the cell biological characteristics of epithelial cancer cell populations in humans whenever possible. The research projects in this program have the modest but important goal of trying to obtain methods for improving decision making about tumor populations.

Proposed Course of Research

Because of the limited resources available and the large number of projects described above, progress will of necessity be piecemeal. Studies 2 and 3 are essentially new studies that have only begun, but will continue as resources allow.

Publications

Cottler-Fox, Ryd, W., Hagmar, B., and Fox, C. H.: Adhesion of metastatic and non-metastatic carcinoma cells to glass surfaces. Int. J. Cancer 26: 689-694, 1980.

Fox, C. H. and Piekarski, L.: Biological manifestations of cancerogenesis, in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05262-01 LB

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Eukaryotic Gene Regulation and Gene Transfer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D.H. Hamer Senior Staff Fellow LB NCI

COOPERATING UNITS (if any) Dr. Moshe Yaniv, Institut Pasteur, Paris, France
Dr. Michael Zasloff, NIAMD, NIH
Dr. Stuart Orkin, Children's Hospital Medical Center, Boston, Massachusetts

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have constructed simian virus 40 (SV40) recombinants carrying a variety of chromosomal eukaryotic genes. These recombinants, together with a complementing helper virus, have been introduced into cultured monkey cells where they replicate to levels of 100,000 copies per cell. Such experiments allow us to examine regulatory sequences, such as promoters and splicing sites, and also to produce substantial quantities of useful gene products, such as human growth hormone and hepatitis B surface antigen, in cultured cells.

PHS-6040
(Rev. 2-81)

Objectives

We wish to understand the regulated expression of animal cell genes.

Methods Employed

Our general strategy is to use SV40 or other vectors to introduce the gene of interest, and mutants derived from it, into cultured cells where their expression can be studied in detail.

Major Findings

1.) Transcription Initiation

We have shown that a mouse α-globin gene promoter is accurately recognized in monkey cells infected with an SV40-globin recombinant (Hamer, Kaehler and Leder, 1980). We are mapping the responsible sequences by analyzing the transcription of deletion mutants lacking various portions of the mouse gene. Recent results indicate that at least one critical control region is contained within the interval 18 to 309 base pairs upstream from the gene.

2.) Splicing and Stable RNA Formation

Our observation that splicing is a pre-requisite for the formation of stable SV40-β-globin hybrid transcripts (Hamer and Leder, 1979) raised several questions about the role of processing in mRNA biosynthesis. First, is the effect of splicing exerted at the transcriptional or the post-transcriptional level? We have been approaching this question by pulse-labelling experiments and by examining the 5' ends of spliced and unspliced primary transcripts. Second, is the splicing requirement a general feature of discontinuous genes? To approach this we have extended our experiments to include a human α-thalassemia gene containing a pentanucleotide deletion at a splice donor site and various constructed human growth hormone gene mutants containing 4, 1 or 0 intervening sequences. Third, how are unspliced RNAs, such as histone mRNA, stabilized and transported? Our working hypothesis is that such RNAs contain "signal sequences" that fulfill the role normally played by splicing sequences. To test this we have inserted a complete mouse histone H4 gene, and fragments derived from it, into an SV40 vector containing no viral splice junctions. The ability of this recombinant to produce stable SV40-histone transcripts suggests that the H4 gene contains such a sequence.

3.) Regulation of Metallothionein Expression

Most of the genes which we have concentrated on to date are "specialized" genes; e.g. globin genes, which are expressed only in erythroid cells, and growth hormone genes, which are active only in the pituitary. Not surprisingly, the expression of these genes is not regulated in monkey kidney cells. Therefore, we have turned our attention to a "housekeeping" gene, the mouse metallothionein I gene. The metallothionein synthesis is regulated by Cd^{++}, Zn^{++} and other transition elements. Recently we have found that metallothionein

synthesis in monkey kidney cells can be induced 100-fold by Cd^{++}. In an attempt to recreate this regulatory system we inserted a mouse metallothionein gene into SV40 and used this recombinant to infect monkey cells in the presence or absence of cadmium. The results show that the cloned gene is induced by cadmium and that the induction is due to an increased rate of transcription.

4.) Protein Production

In addition to these "basic" problems we are interested in using SV40 vectors for the more practical purpose of producing useful animal cell proteins. Our hope is that the SV40-monkey cell system will provide an alternative to the various bacterial systems in those cases where synthesis of the biologically active gene product requires post-translational modification; e.g., amino-terminal processing, glycosylation, secretion, or assembly into particles. Our recent success in producing hepatitis B virus surface antigen (Moriarty et al., 1980) and two human growth hormones argues that this will be the case.

Publications

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Hamer, D.H.: Eukaryotic gene transfer and the new genetics. Am. J. Trop. Med. Hyg. 29: 1093-1095, 1980.

Moriarty, A.M., Hoyer, B.H., Wai-Kuo Shih, J., Gerin, J.L., and Hamer, D.H.: Expression of the hepatitis B virus surface antigen gene in cell culture by using a simian virus 40 vector. Proc. Natl. Acad. Sci. USA 78: 2606-2610, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08701-07 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Nucleic Acid Structures Involved in the Regulation of Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Rosenberg	Research Chemist	LB NCI
	D. Schümperli	Guest Worker	LB NCI
	K. McKenney	FAES Graduate Program	Johns Hopkins University
	Y. Ho	Visiting Fellow	LB NCI
	T. Vogel	Visiting Scientist	Weizmann Institute
	A. Shatzman	IPA	Georgetown University
	D. Sobieski	Chemist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
Cellular Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.0	7.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) We have been investigating nucleic acid structure involved in the control of transcription initiation and termination as well as in translational expression of various RNAs. Using recombinant DNA techniques we have been able to examine the relationship between regulatory function and DNA sequence in a variety of cellular and viral operon control regions. The characterization of mutants which affect both transcriptional and translational expression in these regions has afforded information on the precise molecular events which lead to control element function.

We have used a variety of defined prokaryotic genes to examine the requirements for translation in both eukaryotic cells and cell-free systems.

Recombinant DNA techniques have been used to develop plasmid, phage and bacterial vector systems which allow the isolation, characterization, and comparison of prokaryotic transcriptional regulatory signals. Using this system large amounts of a λ-phage regulatory protein have been isolated.

Efficient expression of the E. coli galactokinase gene attached to a mammalian virus vector has been obtained within mammalian cells.

Project Description:

Objectives:

1. To examine the relationship between function and DNA structure involved in the control of transcription initiation and termination of RNA synthesis, as well as in the translational expression of these mRNAs in both prokaryotic and eukaryotic systems.

2. To examine the nucleic acid structure and function of IS2 type moveable insertion elements found in the bacterial genome.

3. To investigate the ability of defined bacterial and phage mRNAs to be efficiently translated in eukaryotic cells and cell-free systems.

4. To develop new techniques which can be applied to both these studies and to the general examination of nucleic acid structure in other biological systems.

Methods Employed:

Purification of specific RNA transcripts synthesized either in vivo or in vitro; nucleic acid sequence analysis of both RNA and DNA; primed extension of RNA transcription products with deoxyribonucleotides; isolation of protein factors involved either in the processes of transcriptional termination or post-transcriptional modification of RNA; isolation of purified DNA restriction enzymes and their employment in obtaining specific purified fragments of both viral and cellular DNAs; recombinant DNA techniques.

Major Findings:

1. The nucleic acid structure of the 3'-terminal regions of a number of RNA transcripts synthesized in vitro form λ and λgal transducing phages are being examined. These RNAs represent both independently terminated transcription products as well as transcripts requiring the protein factor, Rho, for their termination. Sequence analysis at the 3'-ends of some of these products indicated:

a) that all of the transcripts examined possess similar degrees of 3'-terminal sequence heterogeneity which consisted predominantly of the addition of 1 to 5 adenylate residues to the 3'-terminus of the transcript and

b) that rho factor enhanced termination results in a definite structural change in the nucleotide sequence with which an RNA molecule can terminate.

In addition, we have developed a new methodology for examining the nucleic acid structure in the untranscribed portion of the DNA immediately adjacent to the 3'-terminus of an RNA transcript. This technique has been applied to the determination of sequence information throughout the entire termination region of these transcription products. Little structural homology is apparent in the template DNA beyond the 3'-ends of these transcripts. The lack of homology suggests that this region might not be important to the

termination process. Thus, the majority of the encoded termination signal is transcribed into RNA.

We determined the DNA sequence surrounding and examined termination at a totally rho dependent site (t_{R1}) in bacteriophage λ. The DNA composition at t_{R1} is 75% AT base pairs and termination occurs heterogeneously over four adjacent base pairs. No GC rich or U rich sequence is found at the 3'OH end of the RNA. This sequence is quite different from other transcripts that terminate independently of Rho. However, there is a stable base-paired stem and loop structure near the end of this RNA.

In the absence of Rho factor with normal transcription conditions (i.e. 37° and high triphosphate levels), RNA polymerase undergoes a substantial pause at this site. A mutation, cnc, that interferes with base pairing in the stem and loop, has two effects on transcription: RNA polymerase does not pause in the absence of Rho and transcription does not terminate in the presence of Rho. This suggests that the stem and loop induces RNA polymerase to pause, and that a paused polymerase is important for Rho action.

We have now completed a far more extensive characterization of the entire intercistronic region in which this termination site is positioned. A number of related transcriptional and translational regulatory elements have been defined in this region. More mutations affecting terminator function have been characterized. In addition, we have defined a site (NutR) located immediately preceding the terminator which is apparently involved in anti-termination function. We have shown that the viral function, N, acts in some way to prevent termination (i.e. anti-terminate) at the t_{R1} site thereby allowing for the quantitative and temporal control of transcription through this intercistronic region (i.e. transcription attenuation). Our data indicate that the NutR site comprises an ~17 nucleotide sequence which displays a hyphenated two-fold rotational symmetry. This site is in some way involved in N protein recognition prior to the action of N at the various downstream termination sites.

2. The galactose operon of E. coli is subject to both negative control by gal repressor and positive control by cAMP and its receptor protein (CRP). We have sought to define the nucleotide sequence of the promoter-operator sites responsible for gal regulation. Analysis of transcripts of restriction fragments combined with direct DNA sequencing enabled us to derive the sequence for the gal regulatory region. Cleavage by restriction endonuclease Hinf abolishes cAMP-CRP dependent but not CRP independent transcription. Formation of a cAMP-CRP dependent preinitiation complex prevents cleavage by Hinf. Thus the region upstream of the Hinf site is important for cAMP-CRP stimulation of transcription. This region contains sequence similarities with the CRP recognition site of lac. One gal operator constitutive mutant is resistant to Hinf cleavage suggesting the operator may include at least part of the Hinf site. Further analyses are required to confirm this possibility.

In addition, we have been using an RNA-dependent E. coli S30 translation system to examine the translational efficiencies of in vitro synthesized mRNAs. Because the products of both the transcription and translation reactions can be separately quantitated, the amount of protein produced per mole of a specific mRNA can be determined. Using this method, we have compared the relative translational efficiencies of two different mRNA transcripts of the E. coli galactose operon: the CRP-cAMP dependent mRNA (P1) and the CRP-cAMP independent mRNA (P2). Our results show that the P2 mRNA translates epimerase, the 5' proximal gene product of the gal operon, 4x more efficiently than does the P1 transcript, while the 5' distal gene product, kinase, is translated with equal efficiency. Since the P2 transcript differs from the P1 transcript only by the addition of 5 nucleotides at the 5' terminus, and these nucleotides are outside of the ribosome binding region for epimerase, the selective difference in the translational efficiency of epimerase may be mediated by RNA conformation. It is known that in cells deficient in cAMP, the ratio of epimerase to kinase is about 4x higher than in cells containing cAMP - a phenomenon called discoordinate expression. Moreover, cells deficient in cAMP are thought to produce only gal P2 mRNA, whereas cells with cAMP produce only P1 mRNA. Thus, discoordinate expression is explained by our observation that the P2 transcript produces 4x more epimerase, but a similar amount of kinase, then does the P1 transcript.

3. Earlier work utilizing defined bacterial and phage mRNAs transcribed in vitro from a variety of lambdoid phages, investigated the relationship between the modification of 5' triphosphate end of these transcripts with a 7-methyl-guanosine moiety (i.e. "cap" structure) and the translational efficiency of these prokaryotic mRNAs in wheat germ cell-free extracts. Our results demonstrated an almost absolute requirement for the "cap" structure to obtain efficient translation of the prokaryotic transcripts. All the structural information necessary for proper and efficient recognition and translation of prokaryotic mRNAs using eukaryotic components is encoded in the prokaryotic transcript except for the presence of the 5' 7-methyl-guanosine modification. This implies strong evolutionary constraints on the RNA structure which is used for ribosome recognition and translation initiation, as well as similar constraints on those parts of the ribosome which must interact with this RNA structure.

More recently we have been able to achieve efficient expression of the E. coli galactokinase gene directly within mammalian cells. High levels of the bacterial enzyme galactokinase (galK) are produced in a variety of mammalian cells transfected with an SV40-plasmid recombinant vector carrying the E. coli galK gene. On this vector, the galK coding sequence was inserted downstream from the SV40 early promoter so that the translation start codon for galK became the first AUG on the transcript. To ensure proper transcript maturation, SV40 regulatory sequences for RNA splicing and polyadenylation were included beyond the galK coding region. Cells transfected with this vector produce large amounts of a new galK activity which is similar, by starch gel electrophoresis, to that found in E. coli.

4. The lambda cII gene product is known to be required in vivo for transcriptional activation of both the lambda repressor gene (cI) and the integrase function (int). This activation is essential for phage lysogenic development.

We have selectively cloned the lambda cII gene onto a pBR322 derivative such that cII expression is under the control of the lambda P_L promoter. Transformation of this plasmid into certain bacterial hosts results in high level expression and accumulation of the cII product (~1% of total cellular protein). This single polypeptide product was purified to > 95% homogeneity (in mg quantities).

Standard in vitro transcription reactions were then carried out in the presence and absence of the purified cII protein using as templates appropriate lambda DNA fragments which contain the promoter sites for repressor (P_{RE}) and integrase (P_I) synthesis. Polyacrylamide gel and fingerprint analyses were used to characterize the RNA transcription products and their 5' start-sites. The results indicate that cII protein alone allows RNA polymerase to bind selectively and initiate transcription from these two promoter sites. In the case of the P_{RE} promoter, cII dependent polymerase binding and transcription were eliminated by using DNA templates which contained various cY point mutations.

5. We have developed a recombinant vector system which can be used to isolate, compare and characterize essentially any promoter or terminator signal recognized by E. coli RNA polymerase. The system utilizes plasmid, phage and bacterial vectors and provides extreme flexibility in that any construction made with the plasmid vector can be readily transferred to the phage vector or into the bacterial chromosome. Thus the regulatory signal of interest can be studied in both multiple and single copy.

Future Course:

Our ability to obtain expression of an easily assayable gene product from a structurally defined fusion vector now permits studies of: (1) functional complementation by the bacterial enzyme of primary cells isolated from human patients with galK-deficiency galactosemia or other galK-deficient mammalian cells; (2) transcriptional regulaton in mammalian cells by fusing various eukaryotic promoters to the galK gene; and (3) translational regulation in mammalian cells by selectively altering the primary structure of the 5' non translated portion of the transcript. Other studies using the purified cII protein will be carried out.

The recombinant vector system designed for the study of promoter and terminator sequences will be used to study the effects of various ancillary factors in transcription regulation.

Cancer Research:

Understanding the mechanism by which gene expression is regulated is central to an understanding of cancer.

Publications:

Wulff, D.L., Beher, M., Izumi, S., Beck, J., Mahoney, M., Shimatake, H., Brady, C., Court, D., and Rosenberg, M.: Structure and function of the cY control region of bacteriophage lambda. J. Mol. Biol. 138: 209-230, 1980.

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McKenney, K., Shimatake, H., Court, D., Schmeissner, U., Brady, C. and Rosenberg, M.: A system to study promoter and terminator signals recognized by E. coli RNA polymerase. In Chirikjian, J.G. and Papas, T. (Eds.): Gene Amplification and Analysis, Vol. II: Analysis of Nucleic Acids by Enzymatic Methods. Elsevier-North Holland, in press, 1981.

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Rosenberg, M., McKenney, K., Schümperli, D.: Using the bacterial galactokinase gene to study prokaryotic and eukaryotic promoters. In Chamberlin, M. and Rodriguez, R.L. (Eds.): Promoters: Structure and Function. Praeger Scientific Publishing Co., in press, 1981.

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SUMMARY STATEMENT
LABORATORY OF MOLECULAR BIOLOGY
DCBD, NCI
October 1, 1980 through September 30, 1981

Research in the Laboratory of Molecular Biology is foucused on understanding the factors that control gene expression in animal cells and bacterial cells and to use this information to understand the biochemical basis for the abnormal behavior of cancer cells.

Office of the Chief:

Transformation of fibroblasts by Rous sarcoma virus produces alterations in cell shape, growth, and metabolism. Of all the transformation systems that are under investigation this one has been the best characterized and has been the focus of interest in the Laboratory of Molecular Biology. Transformation by Rous sarcoma virus is due to the action of a single gene src which codes for a 60,000 molecular weight protein. To understand the mechanism of action of this protein, N. Richert and D. Blithe have purified this protein from Rous sarcoma virus induced tumors. From 500 µg of tumor about 50 µg of highly purified protein has been obtained and biochemical studies on the action of this protein have been initiated. It was previously shown by M. Willingham and I. Pastan that the transforming protein is concentrated at the plasma membrane of cells and is not present in significant quantities in the nucleus. Therefore, the src protein probably initially affects the plasma membrane generating a signal that instructs the nucleus to begin DNA synthesis and to alter activity of some of its genes. Two important genes whose activity is diminished are those for fibronectin and collagen. These genes code for diminished adhesion of these cells. The genes for collagen have been cloned in E. coli and these clones are now being used for studies of gene activity in normal and transformed cells. In addition, K. Yamada and coworkers (see below) are studying the mechanism by which fibronectin controls cell adhesion.

In addition to studying the protein responsible for transformation and the genes affected by transformation a program has been initiated to isolated mutant cells which contain host mutations that affect the transformation process. C. Roth and Michael Gottesman have transformed CHO cells with Rous sarcoma virus and isolated from this population revertant cells that have regained normal growth control and normal shape. Some of these revertants have lost the transforming virus; others may have alterations in host functions that suppress the transformation process.

Although analysis of the collagen gene was begun to understand how collagen synthesis is regulated in transformation, the collagen gene family has turned out to be extremely interesting in its own right. B. de Crombrugghe and colleagues have isolated overlapping genomic clones containing the entire gene for pro-alpha$_2$ collagen. One remarkable feature of that gene is that much of the gene is made up of exons of an

identical size of 54 base pairs. This finding strongly suggests that the ancestral gene for all collagens was assembled by amplification of this single genetic unit. Another striking feature of the gene is that in the promoter region there are three large mutually overlapping dyads of symmetry which precede the start site by 40-100 base pairs. These repeats could constitute binding sites for regulatory proteins. There are a number of human diseases in which collagen synthesis is abnormal. B. de Crombrugghe with Visiting Professor David Rowe have begun to isolate and study collagen genes in human cells. This should make it possible to investigate soon human cells which have altered collagen production.

Last year T. Yamamoto isolated a recombinant clone containing the promoter region for Rous sarcoma virus and showed this promoter was active in a cell-free transcription system. J. Sivaswami Tyagi is using this clone to identify and purify factors from extracts of chicken cells that are involved in regulation of RSV transcription. G. Merlino is performing similar experiments using DNA containing the collagen promoter region to look at factors that regulate collagen expression.

Another approach to analyze gene activity is to construct by recombinant DNA techiques eucaryotic vectors that will function both in E. coli and in mammalian cells and therefore can be used to isolate and define eucaryotic genetic segments. B. Howard and colleagues have been working with a vector, pSV2, which contains an origin of replication and an ampicillin resistant marker from E. coli plasmid PBR 322 and a modified SV40 early transcription region. They have inserted into this vector (a) the dihydrofolate reductase coding sequence from E. coli, (b) the xanthine-guanine phosphoribosyl transferase coding sequence from E. coli, (c) the chlorampheical acetyl transferase coding sequence from E. coli, (d) the galactokinase coding sequence from E. coli and other genes. These genes have been amplified in E. coli and the DNA used to transform animal cells and to examine expression of these genes. Recent studies indicate that these genes can be expressed in animal cells and analysis of regulation of their expression is currently under study. Construction of such vectors is clearly in its early stage and is a field which will expand greatly in the next few years because this approach offers not only useful techniques for analysis of gene fuction but perhaps also for therapy of certain human diseases.

In the Molecular Cell Genetics Section Michael Gottesman and coworkers have concentrated on the use of mutational and biochemical analysis of cultured mammalian cells to study some aspects of the behavior of these cells. Specifically, the mechanism of actin of cyclic AMP and regulation of its metabolism, and the means by which the Rous sarcoma virus src gene transform cells have been studied using Chinese hamster ovary (CHO) cells. Temperature-sensitive CHO mutants affecting alpha and beta-tubulin, mutants affecting the regulation and structure of cyclic AMP dependent protein kinase, and mutants resistant to the src gene product of Rous sarcoma virus have been isolated and characterized. These mutants define the steps involved in cyclic AMP and src function and demonstrate the complexity of these pathways. G. Smith and coworkers have shown that

hormone-mediated functions of mammary epithelium requires DNA synthesis, yet is independent of cytokinesis, also the pathway of transformation of mammary epithelium has been shown to be different from the pathway of MMTV-induced transformation. G. Johnson and coworkers have analyzed NAD and 1-methylnicotinamide in several clones of normal and transformed normal rat kidney cells and shown their intracellular levels were found not specifically altered by transformation and did not correlate with the rate of cell division.

S. Wickner who has previously studied DNA replication in procaryotic organisms has initiated studies on another transforming virus, SV40 to understand the steps involved in the regulation of the growth and replication of this virus.

Fibronectin is a major adhesive protein of fibroblasts and some other types of cells. K. Yamada and coworkers in the Membrane Biochemistry Section have been examining its structural domains by cleaving it with proteases and have identified previously regions of fibronectin that bind to collagen and to the plasma membrane. They have now found a domain of 27,000 daltons that binds to actin and a domain of 50,000 daltons that binds to heparin. Thus fibronectin appears to act in different biological and binding events by means of different combinations of these specific domains. It has also been found that fibronectin is present at the "close" adhesive contact regions of cells and not the "focal" adhesive sites. This indicates a role for fibronectin in cell movement and not in cell immobilization. Fibronectin also has a role in embryonic development of both a positive and negative nature depending upon the system.

Cultured cells have on their surface specific receptors for growth promoting substances, hormones, and other ligands. M. Willingham, I. Pastan, and coworkers have been investigating the fate of ligands that bind to the cell surface receptors. In normal fibroblastic cells most receptors are diffusely distributed in the plasma membrane. After ligand binding the receptor-ligand complexes diffuse about in the membrane until they are trapped in special coated regions of the membrane called clathrin-coated pits. These pits which occupy about 1% of the plasma membrane apparently selectively recognize and trap ligand receptor complexes but usually not unoccupied receptors. After the ligand receptor complexes have clustered in pits, they are transferred into a unique vesicle called a "receptosome" which appears to bud off from the neck of a coated pit. This has been demonstrated by direct visualization at the electron microsocpic level and confirmed by injecting antibodies to clathrin, the protein of the coated pit, and showing that coated pits are stable structures associated with the plasma membrane. The coated pit receptosome pathway is responsible for the internalization of $\alpha_2$macroglobulin, insulin, epidermal growth factor, triiodothyronine (J. Cheng) low density lipoprotein, vesicular stomatitis virus and other viruses and ligands. In most cases the ligand is transported by saltatory motion to the region of the Golgi and then transferred to lysosomes. The function of the pathway is to internalize ligands and send them to specialized regions of the cell without allowing them to be rapidly transferred to lysosomes and destroyed.

A number of compounds have been found to inhibit internalization by this pathway; many of these are inhibitors of the enzyme transglutaminase, this finding has led to the hypothesis that clustering of some ligand-receptor complexes in coated pits is dependent upon the activity of a transglutaminase-like enzyme in the pits. Efforts to isolate this enzyme are currently underway.

S.H. Wollman and coworkers in the Cell Organization Section have been investigating the properties of thyroid epithelial cells maintained in suspension culture. They have found that the follicles invert when maintained in 5% serum and this inversion can be prevented and the cells maintain normal polarity by the addition of acid soluble collagen to the medium.

Members of the Biochemical Genetics Section and Developmental Genetics Sections have been studying the molecular mechanisms of the switch from early to late transcription in bacteriophage lambda growth and have found that high level of transcription from the early P_R' promoter prevents access of the transcription proteins to the phage late promoter P_R' that is located downstream, and thus occludes the P_R' activity. The phage Cro protein slowly builds up and represses P_R. This repression thereby turns on the initiation of transcription from the late promoter.

They have also shown that the translation proteins L, S10, and L11 are involved in transcription antitermination reactions of phage lambda. In addition, the E. coli protein, NusA, appears to act as a transcription termination factor.

To study the regulation of the regulatory proteins themselves, e.g., transcription termination factor Rho, adenylate cyclase and cyclic AMP receptor protein (CRP), they have cloned the rho gene and are in the process of cloning the genes for the other two. They have shown that the level of the Rho protein in E. coli is modulated by cyclic AMP and CRP.

They have found that the excision of prophage lambda in vitro requires the same two host proteins (HimA and Hip) that are required for phage integration. These host proteins are also required for the synthesis of the lambda control protein, cII. Two phage protein, Int and Xis are required for excision; the latter inhibits the integration reaction. The control of mucopolysaccharide synthesis in E. coli is under the control of the lon gene. This control is probably mediated through a gene located near min 89 on the E. coli chromosome.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08000-11 LMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of Gene Activity by Cyclic Nucleotides and Transforming Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ira Pastan, Chief	
	Laboratory of Molecular Biology	LMB NCI
	Benoit de Crombrugghe, Head, Gene Regulation Section	LMB NCI
	Michael Gottesman, Head, Molecular Cell Genetics Section	LMB NCI
Others:	Tadashi Yamamoto, Visiting Fellow	LMB NCI
	Glenn Merlino, Guest Worker	LMB NCI
	Jaya Sivaswami Tyagi, Research Fellow	LMB NCI
	Charles Roth, Expert	LMB NCI
	Toolsee Singh, Visiting Fellow	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.4	4.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

cDNA clones of α_1 and α_2 Type I collagen and fibronectin and genomic clones of α_2 collagen have been isolated. These have been used to study how RSV transformation controls gene expression. A clone containing the promoter of pro α_2 collagen has been isolated and sequenced. In vitro transcription of the collagen gene has been obtained with this clone. Mutant CHO cells unresponsive to cyclic AMP treatment have been found to have defects in Type I regulatory subunit and Type II protein kinase.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08000-11 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Regulation of Gene Activity by Cyclic Nucleotides and Transforming Proteins.

Previous Serial Number: ZO1 CB 08000-10 LMB

Principal Investigators: Ira Pastan, Benoit de Crombrugghe, Michael Gottesman

Other Investigators: Tadashi Yamamoto, Glen Merlino, Jaya Sivaswami Tyagi, Charles Roth, Toolsee Singh

Man Years: October 1, 1980 through September 30, 1981

Total:	5.4
Professional:	4.4
Others:	1.0

Project Description:

Objectives: To understand how cyclic AMP and transforming proteins control gene activity and the behavior of cultured cells.

Methods Employed: Prepare cloned cDNAs and genomic DNA to use to study messenger RNA synthesis in intact cells or cell-free systems. Isolate animal cells that have mutations in the enzymes of cyclic AMP metabolism and RSV expression and determine the biochemical basis of these alterations.

Major Findings: DNA from a recombinant plasmid containing one Long Terminal Repeat (LTR) of Rous Sarcoma virus has been used to investigate the nature of initiation of RSV transcription. Using a transcription system containing RNA polymerase II, T. Yamamoto has shown that *in vitro* transcription begins at the site corresponding to the 5' end of mature 39S RNA (virion RNA). Initiation of transcription begins 23bp downstream from a "promoter-like" sequence TATTTAAG. This result indicates the basic information necessary for RSV transcription lies within the viral genome.

A genomic clone containing the promoter region of Type I α_2 collagen was isolated from chicken DNA. Using this clone G. Merlino has done experiments in a cell-free system and shown that RNA polymerase II initiates transcription at a site that corresponds to the site of initiation *in vivo*. This site lies 33bp downstream from the sequence TATAAATA and about 80 bp downstream from the sequence GCCCATT. This CAT sequence lies within three large and mutually exclusive dyads of symmetry which could participate in regulation of the gene.

J. Sivaswami Tyagi has prepared a cell-free transcription system requiring purified calf thymus RNA polymerase II and is examining factors that regulate RSV and collagen transcription.

C. Roth has isolated RSV transformed Chinese hamster ovary cells and flat non-transformed revertants of these transformed cells. With Dr. Tyagi he has characterized these revertants and shown some have lost the entire viral genome and others have retained the virus but the virus has undergone some change that prevents src expression. A search for host mutants restricting RSV expression is still underway.

Michael Gottesman has isolated a large variety of CHO cells unresponsive to cyclic AMP. In general these cells carry mutations in cyclic AMP dependent protein kinases. T. Singh has characterized a number of these and shown that one group of mutants has a defect in Type I regulatory subunit and another lacks Type II protein kinase.

Significance for Cancer Research and the Program of the Institute: The invasive properties of tumor cells may be related to alterations in collagen, fibronectin and cyclic AMP. Understanding how the synthesis of these molecules is altered in transformation may suggest new methods of cancer treatment.

Publications:

Yamamoto, T., Sobel, M.E., Adams, S.L., Avvedimento, V.E., DiLauro, R., Pastan, I., de Crombrugghe, B., Showalter, A., Pesciotta, D., Fietzek, P., and Olsen, B. Construction of a recombinant bacterial plasmid containing pro-α1 (I) collagen DNA sequences. J. Biol. Chem. 255: 2612-2615, 1980.

Gottesman, M.M., LeCam, A., Bukowski, M., and Pastan, I. Isolation of multiple classes of mutants of CHO cells resistant to cyclic AMP. Somat. Cell Genetics 6: 45-61, 1980.

Yamamoto, T., Tyagi, J.S., Fagan, J.B., Jay, G., de Crombrugghe, B., and Pastan, I. Molecular mechanism for the capture and excision of the transforming gene of avian sarcoma virus as suggested by analysis of recombinant clones. J. Virology 35: 436-443, 1980.

Vogeli, G., Avvedimento, E.V., Sullivan, M., Maizel, J., Lozano, G., Adams, S.L., Pastan, I., and de Crombrugghe, B. Isolation and characterization of genomic DNA coding for pro-α2 collagen. Nucl. Acids Res. 8: 1823-1837, 1980.

Fagan, J.B., Pastan, I., and de Crombrugghe, B. Sequence rearrangement and duplication of double stranded fibronectin cDNA probably occurring during cDNA synthesis AMV reverse transcriptase and Escherichia coli DNA polymerase I. Nucl. Acids Res. 8: 3055-3064, 1980.

Fagan, J.B., Sobel, M.E., Yamada, K.M., de Crombrugghe, B., and Pastan, I. Effects of transformation on fibronectin gene expression using cloned fibronectin cDNA. J. Biol. Chem. 256: 520-525, 1981.

Showalter, A.M., Pesciotta, D.M., Eikenberg, E.F., Yamamoto, T., Pastan, I., de Crombrugghe, B., Fietzek, P., and Olsen, R. Nucleotide sequence of a collagen cDNA-fragment coding for the carboxyl end of Pro 1 (1)-chains. FEBS Letters 111: 61-65, 1980.

Avvedimento, V.E., Vogeli, G., Yamada, Y., Maizel, Jr., J.V., Pastan, I., and de Crombrugghe, B. Correlation between splicing sites within an intron and their sequence complimentary with U_1 RNA. Cell 21: 689-696, 1980.

Ohkubo, H., Vogeli, G., Mudryj, M.J., Avvedimento, V.E., Sullivan, M., Pastan, I., and de Crombrugghe, B. Isolation and characterization of overlapping. Proc. Natl. Acad. Sci. USA 77: 7059-7063, 1980.

Avvedimento, E., Yamada, Y., Lovelace, E., de Crombrugghe, B., and Pastan, I. Decrease in the levels of nuclear RNA precursors for α_2-collagen in Rous sarcoma virus transformed fibroblasts. Nucl. Acids Res., in press, 1981.

Vogeli, G., Ohkubo, H., Avvedimento, V.E., Yamada, Y., Mudryj, M., Pastan, I., and de Crombrugghe, B. A repetitive structure in the chick α_2-collagen gene. Cold Spring Harbor, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08001-11 LMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Cyclic AMP and Transforming Viruses in the Regulation of Cell Behavior

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ira Pastan, Chief, Laboratory of Molecular Biology	LMB NCI
	Mark Willingham, Head, Ultrastructural Cytochemistry Section	LMB NCI
	Michael Gottesman, Head, Molecular Cell Genetics Section	LMB NCI
Others:	Nancy Richert, Expert	LMB NCI
	Diana Blithe, Guest Worker	LMB NCI
	Charles Roth, Expert	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.7	2.7	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To learn how Rous sarcoma virus transforms cells we have purified the transforming protein from tumors. We are currently characterizing the highly purified protein and assessing its effect when injected into normal cells.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08001-11 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1980

Project Title: Role of Cyclic AMP and Transforming Viruses in the Regulation of Cell Behavior

Previous Serial Number: ZO1 CB 08001-10 LMB

Principal Investigators: Ira Pastan, Mark Willingham, and Michael Gottesman

Other Investigators: Nancy Richert, Diana Blithe, and Charles Roth

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	3.7
Professional:	2.7
Other:	1.0

Project Description:

Objectives: To understand the role of cyclic AMP, hormones and viral gene products in malignant transformation and particularly how these factors regulate the growth, morphology, and other properties of cultured fibroblastic cells.

Methods Employed: Cell culture, viral transformation and standard biochemical analyses of the enzymes involved in cyclic AMP metabolism and the synthesis of macromolecules. Preparation of antibodies against tumors induced by Rous sarcoma virus. Purification of proteins.

Major Findings: The transforming protein from RSV induced rat tumors has been purified about 1,000 fold by N. Richert and D. Blithe. They will use this material to investigate the mechanism by which this protein transforms cells.

C. Roth has studied the effect of cyclic AMP on RSV transformed CHO cells. He finds these cells are less responsive to the changes in cell shape and slowing of cell growth then cells that are spontaneously transformed. Since the RSV transformed cells have normal cyclic AMP dependent protein kinases, his result suggests that RSV affects some more distal step to bring about the decreased cyclic AMP response.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approaches 1, 2 and 5; Objective 4, Approach 2; Objective 6, Approach 3. Various aspects of this work will lead to a better understanding of how cells become cancer cells and how the growth of cancer cells is controlled. It also has therapeutic implications.

Proposed Course:

(1) Further characterization of the transforming protein of RSV.
(2) Search for cellular targets to account for p60^{src} action.

Publications:

Pastan, I., Jay, G., Richert, N., Davies, P.J.A., and Willingham, M. Nature and intracellular location of the product of the src gene of avian sarcoma virus. Cold Spring Harbor, Symposium on RNA Tumor Viruses Vol. XLIV 1023-1029, 1980.

Blithe, D.L., Pastan, I., Buck, C.A., and Warren, L. Carbohydrate groups of glycoproteins in a glycosylation-defective, low adherent mutant mouse cell. Biochemistry International, Vol. 1., No. 1, pp. 71-76, 1980.

Willingham, M.C. and Pastan, I. Regulation of cell growth: The morphologic and growth phenotypes of neoplastic transformation in cultured fibroblasts. in Growth and Growth Factors, Japan Med. Res. Foundation Pub. #12, Univ. of Tokyo Press, Tokyo, Japan, 1980.

Pastan, I., Willingham, M., de Crombrugghe, B. Aging and cancer: Cyclic AMP and altered gene activity. Submitted JNCI-Monograph, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08006-10 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Control of Gene Expression in Bacteriophage Lambda

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Max E. Gottesman Head, Biochemical Genetics Section	LMB NCI
Others:	Douglas Ward, Visiting Fellow	LMB NCI
	Amos Oppenheim, Visiting Scientist	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are continuing our study on the nature of transcription termination in E. coli and of the mechanism of action of the bacteriophage lambda antitermination-function, the product of the lambda N-gene.

In addition, we are studying the properties of the lambda transcription initiation function, the product of gene cII.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08006-10 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Control of Gene Expression in Bacteriophage Lambda

Previous Serial Number: ZO1 CB 08006-09 LMB

Principal Investigator: Max E. Gottesman

Other Investigators: Douglas Ward and Amos Oppenheim

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	3.0
Professional:	3.0
Others:	0.0

Project Description:

Objectives: The lambda gene N-product suppresses transcription termination. Gene cII product stimulates transcription initiation. Our intention is to determine the mechanisms of action of N-function and of cII product.

Methods Employed: Standard microbial genetic and biochemical techniques, as well as recombinant DNA technology.

Major Findings: We have been investigating the mechanism of action of the lambda antitermination function, the N gene-product. Our approach involves studying host mutants that affect N activity. For its action in suppressing transcription termination, the N-gene product of lambda requires the product of the E. coli nusA gene. Starting with an nusA mutant, we have isolated host suppressor mutants in which lambda can produce active N-product. Two such suppressors have been characterized. One is a mutation in nusB which allows lambda phage but not the closely related phage, 21, to grow in nusA mutants. The second resides in the gene encoding the ribosomal large subunit protein, L11. The involvement of a ribosomal cistron in the functioning of N-product suggests that translation may play a role in the antitermination mechanism.

We find also that the nusA1 and nusB5 mutations relieve polarity in E. coli. This suggests that the wild-type products of these genes are transcription termination proteins like Rho.

We find that cII-protein is not made in E. coli himA hosts, and is made, but does not function, in hosts producing large amounts of N-protein.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 1. In cancer cells, the expression of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using λ as a model system. This understanding might help to prevent the conversion of normal cells to those capable of forming cancers.

Proposed course: 1. To study other host mutations that influence the activity of N-gene product. 2. To determine if N-product can function in the absence of translation. 3. To establish a cell-free system for the assay of N-function. 4. To understand the regulation of cII-protein synthesis and activity. 5. To understand the biochemistry of transcription initiation and termination signals.

Publications:

Ward, D.F., and Gottesman, M.E. The nus mutations affect transcription termination in E. coli. Nature, in press, 1981.

Greenblatt, J., Li, J., Adhya, S., Friedman, D., Baron, L., Redfield, B., Kung, H., and Weissbach, H. Evidence that the L factor required for DNA dependent In vitro synthesis of β-galactosidase is the E. coli nusA gene protein. Proc. Natl. Acad. Sci. USA, 77: 1991-1994, 1980.

Gottesman, M., Adhya, S., and Das, A. Transcription antitermination by lambda N-gene product. J. Mol. Biol., 140: 57-75, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08010-07 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Morphologic Mechanisms of Motility and Neoplastic Transformation in Culture.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mark C. Willingham	Chief, UC Section	LMB	NCI
Others:	Ira H. Pastan	Chief, LMB	LMB	NCI
	Jurgen Wehland		LMB	NCI
	Robert B. Dickson		LMB	NCI
	James H. Keen		LMB	NCI
	Claude B. Klee		LB	NCI

COOPERATING UNITS (if any)

Laboratory of Biochemistry, DCBD, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Ultrastructural Cytochemistry Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Neoplastic transformation produces many changes in cell physiology. To study these changes, we have developed and utilized a new ultrastructural immunocytochemical technique (EGS procedure) to localize over twenty different intracellular protein antigens in cultured cells. The most recent of these studies localized the G glycoprotein of Vesicular Stomatitis Virus, the bristle coat protein clathrin, fibronectin and the calcium-regulatory protein calmodulin. We have also recently combined these localization methods with single cell microinjection techniques to study the processes of receptor-mediated endocytosis and exocytosis, showing that clathrin-coated pits mediate the entry of receptor-ligand complexes but do not form isolated coated vesicles, and that the exocytosis pathway from the Golgi does not involve clathrin coated structures. Also, using cytochemical markers, we have shown that coated pits are stable elements on the plasma membrane, and that isolated coated vesicles do not exist in living cultured fibroblasts.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08010-07 LMB
1. Laboratory of Molecular Biology
2. Ultrastructural Cytochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: The Morphologic Mechanisms of Motility and Neoplastic Transformation in Culture.

Previous Serial Number: ZO1 CB 08010-06 LMB

Principal Investigator: Mark C. Willingham

Other Investigators: Ira H. Pastan, Chief, LMB
Jurgen Wehland
Robert B. Dickson
James H. Keen
Claude B. Klee

Cooperating Unit: Laboratory of Biochemistry, DCBD, NCI

Man Years: October 1, 1980 through September 30, 1980

Total:	4.0
Professional:	4.0
Others:	0.0

Project Description:

Objectives: To investigate the mechanisms that control cell motility, endocytosis, exocytosis, intracellular protein traffic, viral infection and the morphologic and growth manifestations of malignant transformation.

Methods: Cell culture, specialized light and electron microscopic morphologic and immunocytochemical methods, including the EGS ultrastructural localization procedure, single cell microinjection techniques, and specialized biochemical purification and analytical methods.

Major Findings: Neoplastic transformation of cultured fibroblasts alters their shape, adhesion to substratum, surface microstructure, organization of cytoskeletal elements, motility patterns, as well as growth properties. These former properties, referred to as "the morphologic phenotype of transformation" are primarily controlled by the regulation of adhesion to substratum combined with surface membrane-associated cytoskeletal elements.

We have previously analysed the location and organization of a number of structural proteins in the cytoplasm in an effort to understand their relationships and effects on the morphologic alterations in transformation.

These studies have interrelated the mechanism of motility, endocytosis, cytoskeletal movements, and exocytosis. Most recently, we have characterized the location of the bristle-coat protein of coated pits, clathrin. These studies have shown that virtually no clathrin could be found in the cytosol at locations away from known coated pit basketwork structures. This suggested that depolymerization of the clathrin structure might be unlikely to occur in living cells. Further, we localized calmodulin, a major regulatory protein in the cytoplasm and found that it was diffusely distributed throughout the cytosol, and no concentrations were found related to coated pits or to microfilamentous elements of the cytoskeleton.

Besides the plasma membrane, clathrin also forms coated pits in the Golgi system, in an area called the GERL (Golgi-endoplasmic reticulum-lysosome) complex which had been suggested to be involved in the processing and delivery of lysosomal enzymes to lysosomes. We found both clathrin and a lysosomal enzyme, β-galactosidase in these regions. Further, the clathrin-coated regions of the GERL had been suggested to be involved in exocytosis, but using the G-glycoprotein of Vesicular Stomatitis Virus as a model exocytic protein, we found that these coated regions are not involved in G-protein exocytosis. A similar finding was also made in analyzing the exocytosis of fibronectin, a secretory protein of fibroblasts. In addition, microinjection of antibodies against clathrin failed to interfere with the exocytosis of G-protein in living cells.

We have also studied the role of clathrin in the endocytic process, using microinjection of anti-clathrin antibody and a new label for $alpha_2$-macroglobulin (a model endocytosed ligand in fibroblats) coupled to colloidal gold. These experiments showed that injected anti-clathrin did not interfere with the endocytic process, nor did they induce any aggregation of clathrin structures in the cytoplasm, in spite of the fact that the same cell could be injected hours later with exogenous clathrin, resulting in large immunoprecipitates forming in the living cell. These results strongly suggested that clathrin-coated pits at the plasma membrane do not pinch off to form isolated coated vesicles. Other experiments, using impermeant electron-dense markers have shown similar findings, in that coated pits at the plasma membrane can be shown to always be in continuity with the cell surface. These results have radically changed the concepts of the mechanisms of entry, exit, and intracellular compartmentation of macromolecules. These processes are of vital significance to the processing of growth-promoting hormones, metabolic macromolecules such as low density lypoprotein and lysosomal enzymes, and pathologic macromolecules such as some toxins (diphtheria toxin, Pseudomonas toxin) and viruses (vesicular stomatitis virus, adenovirus). The manipulation of these entry and exit systems is of particular importance in the control of viral infection and propagation.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 6, Approach 3.

Transformation of cultured cells appears closely linked with the ability to form malignant tumors in vivo. The understanding of the basic mechanisms

that control cell movement, response to growth-promoting factors, virus infectivity, and the morphologic changes that occur following transformation by transforming viruses are likely to be of great value in understanding the basic mechanisms that are altered in most cancer cells. This understanding is likely to have significant impact on the ability to design successful therapeutic procedures.

Proposed Course: We will continue to study to basic cellular morphologic mechanisms that regulate cell movement, entry and exit of cell proteins, viruses, and hormones, and the interactions of specific transformation-linked molecules (src proteins) with these systems.

Through the study of the alterations in cell functions related to transformation, we will try to gain a more specific understanding of the precise mechanisms by which cancer cells are able to grow uncontrollably and metastasize.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08011-07 LMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure and Role of a Transformation-Sensitive Cell Surface Glycoprotein

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth M. Yamada Head, Membrane Biochemistry Section	LMB NCI
Others:	Masao Hayashi, Visiting Fellow	LMB NCI
	Steven K. Akiyama, Guest Worker	LMB NCI
	Tom M.A.R. Dubbelman, Guest Worker	LMB NCI
	Phyllis R. Strauss, Guest Worker	LMB NCI
	Hideyasu Hirano, Visiting Fellow	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Membrane Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.9	4.9	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major cell surface glycoprotein fibronectin is decreased after malignant transformation and is involved in cellular adhesion. We have investigated its mechanism of action. Polypeptide structural domains containing specific binding sites for the plasma membrane, collagen, heparin, and actin have been isolated, purified, and characterized biochemically and in assays for cell adhesion. The immunofluorescence localization of fibronectin was compared with known adhesive structures, and it was found to be present in close, but not in most focal, cell-substrate adhesive contact sites. A positive regulatory role in embryonic differentiation was suggested in experiments with neural crest cells, in which treatment with exogenous fibronectin promoted adrenergic cell differentiation.

Our objectives will be to determine the composition and structure of the multiple active sites of fibronectin, the mechanisms by which this major cell surface protein helps to regulate cell behavior and differentiation, and the structure and function of its gene.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08011-07 LMB
1. Laboratory of Molecular Biology
2. Membrane Biochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Structure and Role of a Transformation-Sensitive Cell Surface Glycoprotein

Previous Serial Number: ZO1 CB 08011-06 LMB

Principal Investigator: Kenneth M. Yamada

Other Investigators: Masao Hayashi, Steven K. Akiyama, Tom M.A.R. Dubbelman, Phyllis R. Strauss, Hideyasu Hirano

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	5.9
Professional:	4.9
Other:	1.0

Project Description:

Objectives: The major cell surface protein fibronectin is depleted after neoplastic transformation. Our objectives are to determine fibronectin's biochemical structure, its role in cell behavior, its mechanism of action, and its regulation.

Methods: Fibronectin is isolated from chick embryo fibroblasts using low concentrations of urea, then further purified by salt fractionation and column chromatography at alkaline pH. Fibronectin is cleaved with various proteases to polypeptide fragments containing specific binding sites. Pronase-, trypsin-, and subtilisin-resistant domains are chromatographed on collagen-, heparin-, or actin-affinity columns in order to isolate the structural domains responsible for binding to each of these ligands. Collagen- and heparin-binding domains are subsequently purified to homogeneity by molecular sieve chromatography, and characterized by previously published bioassays, amino acid sequencing with a sequenator, carbohydrate assays by the phenol-sulfuric acid method, and polyacrylamide gel electrophoresis.

Embryonic avian heart fibroblasts and neural crest cells are cultured on glass substrates or on 3-dimensional collagen gels and are examined for fibronectin localization by immunofluorescence microscopy with affinity-purified antifibronectin antibodies. Adhesive sites are characterized by interference contrast microscopy using light reflected from the ventral surfaces of cells.

Major findings: Fibronectin is thought to function in the interaction of cells with a variety of biologically important macromolecules such as collagen, heparin, and actin. We have explored its mechanism of action by dissecting the molecule into its structural domains with proteases. In addition to the previously isolated domains for binding to collagen and the plasma membrane, we have purified polypeptide domains of 27,000 and 50,000 daltons that contain binding sites of fibronectin to actin and heparin, respectively. Detailed comparisons of the heparin and collagen binding sites reveal substantial differences in biological activity, carbohydrate composition, and amino acid sequence. Fibronectin appears to act in different biological and binding events by means of different combinations of these specific domains.

The locations of fibronectin on the cell surface were compared to those of previously described adhesive structures that mediate the adhesion of cells to substrates. Although fibronectin is generally absent from "focal" adhesive sites, it is present in "close" adhesive contact regions. This localization supports the concept that it is involved in adhesive processes more related to cell movement than to immobilization.

We have continued to investigate the role of fibronectin in embryonic development. Although previous studies showed an inhibitory role in development of cartilage and muscle, a positive regulatory function is suggested with neural crest cells. Treatment of these migratory cells with fibronectin to attempt to mimic an environment they encounter in vivo results in a substantial increase in differentiation of catecholamine-containing cells, suggesting that fibronectin may function in promoting differentiation of adrenergic cells of sympathetic ganglia.

Additional collaborative studies have provided evidence using recombinant DNA methodology suggesting a suppression of fibronectin gene transcription in malignant cells, documented the processing of the carbohydrate chains of fibronectin during biosynthesis, and demonstrated that both cellular and plasma forms of fibronectin can act in the endocytosis of collagen by macrophages.

Significance for Cancer Research and Program of the Institute: National Cancer Plan Objective 3, Approach 5.

The cell surface glycoprotein fibronectin functions in normal cellular adhesion, morphology, and cell-cell interactions, and its loss after transformation partially accounts for the changes in these properties in transformed cells. Recombinant DNA studies suggest that such decreases in part result from altered gene expression. To determine fibronectin's mechanism of action, we have purified and characterized specific active sites from this molecule. Binding to these cell-, collagen-, heparin-, and actin-binding sites can explain how this protein can mediate a variety of adhesive and binding events. Other studies suggest that changes in fibronectin levels during embryonic development can have positive or negative regulatory roles in differentiation. These studies should provide insight into the regulation of embryonic, adult, and neoplastic cell behavior.

Proposed Course: To determine the structure of cell-, collagen-, heparin-, and actin-binding sites of fibronectin, to compare the polypeptide structures of cellular and plasma fibronectins, to identify the membrane component to which they bind on the cell surface, to raise monoclonal antibodies to distinguish between specific domains and the different forms of fibronectin, and to characterize the gene(s) for fibronectin.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08700-09 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Regulation of the α_2 Collagen Gene; Regulation of Gene Expression in Bacteria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Benoit de Crombrugghe	Chief, Gene Regulation Section	LMB NCI
	Ira Pastan	Chief, Laboratory of Molecular Biology	LMB NCI
Others:	Gabriel Vogeli	Senior Staff Fellow	LMB NCI
	Enrico Avvedimento	Visiting Fellow	LMB NCI
	Hiroaki Ohkubo	Visiting Fellow	LMB NCI
	Yoshihiko Yamada	Visiting Associate	LMB NCI
	Maria Mudryj	Chemist	LMB NCI
	Stephen J. W. Busby	Visiting Associate	LMB NCI
	Hiroji Aiba	Visiting Fellow	LMB NCI
	David Rowe	Assistant Professor	U. Conn
	Rodolfo Frunzio	Visiting Fellow	LMB NCI
	Catherine McKeon	Postdoctoral Fellow	LMB NCI

COOPERATING UNITS (if any) Jacob Maizel, NICHD; Margery Sullivan, Genex Corp.; Peter Muller, Max Planck Institute, Munich, Germany; Bjorn Olsen & Peter Fietzek, Rutgers Medical School, New Jersey; Michael O'Neill, University of Maryland, Catonsville, Md.; Bruce Howard & Cory Gorman, NCI; Mark Sobel, NIDR.

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Gene Regulation Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
11.0	9.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. We have isolated and characterized a developmentally regulated eucaryotic gene, the gene for type I α_2 collagen. Its coding information is subdivided in at least 52 exons. The size of the exons, which is remarkably conserved, indicates how the gene was assembled during evolution by multiple duplications of a single genetic unit.

2. We have characterized the promoter of this gene by DNA sequence. The region before the start site contains three large overlapping dyads of symmetry which could constitute binding sites for regulatory proteins.

3. We have constructed recombinant plasmids in which the collagen promoter is placed adjacent to a small bacterial gene, the gene encoding chloramphenicol acetylase, to study the expression of this recombinant in animal cells.

4. We have begun to isolate one of the human collagen genes (α_1 type I) and two other chick collagen genes.

5. We have shown that the two promoters of the galactose operon of E. coli are functional in intact cells and have obtained additional promoter mutants in the E. coli galactose operon which elucidate the complex regulatory mechanisms of this operon.

Serial No. Z01 CB 08700-09 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Regulation of the α_2 Collagen Gene; Regulation of Gene Expression in Bacteria

Previous Serial No.: Z01 CB 08700-08 LMB

Principal Investigators: Benoit de Crombrugghe, Chief, Gene Regulation Section, LMB
Ira Pastan, Chief, Laboratory of Molecular Biology, DCBD

Other Investigators: Gabriel Vogeli, Senior Staff Fellow; Enrico Avvedimento, Visiting Fellow; Hiroaki Ohkubo, Visiting Fellow; Yoshihiko Yamada, Visiting Associate; Maria Mudryj, Chemist; Stephen J. W. Busby, Visiting Associate; Hiroji Aiba, Visiting Fellow; Cathy McKeon, Guest Worker; David Rowe, IPA; Rodolfo Frunzio, Visiting Fellow.

Cooperating Units: Jacob Maizel, NICHD; Peter Muller, Max Planck Institute, Munich, Germany; Bjorn Olsen and Peter Fitzek, Rutgers Medical School, New Jersey; Michael O'Neill, University of Maryland, Catonsville, Maryland; Mark Sobel, NIDR; Bruce Howard, NCI

Man Years: October 1, 1980 through September 30, 1981

Total:	11.0
Professional:	9.0
Other:	2.0

Project Description:

Objective: To understand the precise molecular mechanisms which control the expression of specific genes in animal and bacterial cells.

Methods: Measurement of RNA and protein synthesis in vivo and in cell-free systems; purification of specific DNA fragments and RNA species; nucleotide sequence analysis of DNA and RNA; introduction of segments of animal and bacterial genes in the DNA of bacterial plasmids and bacteriophage; construction of recombinant DNAs; purification of factors active in regulation of gene expression; construction and screening of gene libraries.

Major Findings:

1. The collagen gene.

We have isolated a series of overlapping clones which span the chick $\alpha 2$ (type I) collagen gene, plus its 5' and 3' flanking sequences, by successive screenings of a library of chick genomic DNA fragments. The $\alpha 2$ collagen gene has a length of about 40 kilobases while the corresponding mRNA is 5,000 nucleotides long. The coding information of the gene is subdivided in more than 50 exons interspersed with introns of various sizes. The complexity of this gene implies that the processing of the primary transcript to mature translatable mRNA includes at least 50 splicing events. The splicing reaction must precisely connect the different coding segments in an exact and unique pattern. The first 6 bases at the extreme 5' ends of the introns, immediately adjacent to the exons, are conserved to a much higher degree than in other genes. The sequences at the extreme 3' end of the introns also show a large degree of homology. The sequences at both ends of introns exhibit a very good complementarity with the sequence of the 5' end of U_1 RNA, a small nuclear RNA present in large copy numbers in the nuclei of eucaryotic cells. Other experiments have provided additional evidence for the participation of U_1 RNA in RNA splicing. We have indeed found a correlation between splice sites located inside introns and their sequence complementarity with U_1 RNA.

To try to answer the question of why the coding information of the collagen gene is subdivided in so many exons, we have sequenced a small number of exons in 3 different parts of the gene corresponding to the major, helical portion of the protein. The remarkable finding is that most exons have an identical size of 54 bp although the sequences within these exons vary. This finding strongly suggests that the ancestral gene for all collagens was assembled by amplification of a single genetic unit containing an exon of unique size. Later, during evolution, the sequences within these exons evolved by sucessive point mutations and in some case by deletions or additions of 9 bp or multiples of 9 bp.

Since our major interest is the developmental regulation of the collagen genes, and since this regulation presumably occurs at the level of transcription, we have determined the start site for transcription of the type I $\alpha 2$ collagen gene and characterized the promoter for this gene. The most striking feature of this promoter sequence is the presence of three large mutually overlapping dyads of symmetry in a region of the promoter which preceeds the start site by 120 to 40 bp. These three inverted repeats could constitute binding sites for regulatory proteins. The promoter also contains sequences found in other eucaryotic promoters at similar distance from the start site.

The promoter sequence was also used as template for cell-free transcription with a soluble extract from He La cells. The RNA, which is made *in vitro*, starts at the same place as the RNA made in intact cells.

We have recently begun to examine the degree of methylation of the collagen gene in tissues where the gene is expressed and in tissues where it is not. Preliminary evidence suggests that in several tissues, which are not

believed to synthesize α_2 (I) collagen, the α_2 collagen is not methylated. This apparently violates the hypothesis that in tissues which do not express a certain gene, this gene is more methylated than in tissues which express the gene.

2. Regulation of collagen RNA synthesis.

The levels of collagen RNA are regulated by two types of factors which both have pleiotropic effects: p60^{src}, a protein which causes chick cells to become oncogenic, and phorbol esters, a class of chemicals previously known as tumor promoters. Both factors cause a substantial reduction in collagen RNA levels in chick embryo fibroblasts. We have further shown that p60^{src} decreases the levels of collagen RNA precursors in the nucleus; hence, the control by p60^{src} is likely to be at the level of transcription.

To better study the expressions of the α_2 (I) collagen gene in vivo, we have constructed a recombinant plasmid in which the collagen promoter is placed adjacent to one of two small bacterial genes, the gene for chloramphenicol acetylase (CAT) or the gene for xanthine-guanosine phosphoribosyl-transferase (xGPRT).

Bruce Howard and Cory Gorman have shown that the chloramphenicol acetylase gene is expressed in animal cells when placed next to the SV40 early promoter P. Berg and his colleaques had shown the same for XGPRT. We hope to disect the promoter region of the α_2 (I) collagen gene by introduction of deletions, insertions and point mutations and study the effect of these mutations on the expression of CAT or XGPRT. Since the expression of the α_2 (I) collagen gene is inhibited by the presence of p60^{src}, and as this regulation, although indirect, is probably mediated by a transcriptional control mechanism, we also want to determine the target site on the promoter for this type of control.

3. Isolation of other collagen genes.

David Rowe, a visiting professor from the University of Connecticut, has constructed and identified a cDNA clone for human α_1 collagen. Using this cDNA clone, he has begun to isolate the corresponding human gene. In collaboration with Dr. Rowe, we will isolate the entire gene and begin to study some of the genetically linked human disorders which affect the structure or the regulation of collagen genes. Dr. Rowe has a large collection of cells from patients with such disorders.

We have also begun to isolate the gene for chick type II collagen, a form of collagen mainly synthsized by choncrocytes. This gene apprears also to be controlled by p60^{src}.

We have also isolated a segment of another collagen gene which we have not yet identified but which is not coding for either type I, type II or type III. Our working hypothesis is that this gene might encode type V collagen, a basement membrane collagen.

4. Studies on the regulation of the galactose operon of E. coli.

The regulatory elements of the E. coli galactose operon have a unique arrangement which probably reflects the dual role of galactose in cellular

metabolism. First, there are two overlapping promoters, P_1 and P_2, which control the genes of the galactose operon. One promoter requires cyclic AMP (cAMP) and its receptor protein (CRP), the other functions in the absence of cAMP-CRP and is inhibited by these factors. The two promoters probably ensure a constant basal level of UDP-galactose-epimerase (the enzyme which converts UDP-glucose to UDP-galactose) regardless of the fluctuations in the intracellular cyclic AMP concentrations. The existence of the two promoters was first demonstrated by in vitro transcription of gal DNA fragments. We have now shown that these two promoters are functional in intact cells. In cya- cells, the extreme 5' end of gal RNA contains 5 more nucleotides which are absent in wild-type cells. Hence, in wild-type cells the major gal promoter is P_1, in cya-cells the principal gal promoter is P_2.

The operator of the gal operon is located 60 base pairs preceding the P_1 start site for transcription, in contrast to the location of the operator in other bacterial or bacteriophage systems. In the latter systems, the repressor binds more closely to the respective initiation sites and directly blocks the formation of a stable RNA polymerase-promoter complex.

CRP interacts with gal DNA between 50 and 25 base pairs preceding the P_1 initiation site. Comparison of the sequence of the gal CRP site with that of the lac CRP site (located 50-70 base pairs preceding the lac initiation site), the ara CRP site and, some other CRP sites suggests a common sequence recognized by CRP. We have developed a model in which all CRP sites are recognized by the two subunits of CRP. Some, like the gal site, are monomolecular; some like the lac site are biomolecular.

We have constructed a set of recombinant plasmids in which the lac operon is controlled by the gal promoter. These plasmids were used for site specific mutagenesis experiments: a small DNA fragment was mutagenized and then introduced in the plasmid.

Four new gal promoter mutants were isolated. Two maps in the CRP binding site and abolish P_1 activity. They are also consistent with our model for CRP binding. In one mutant, a deletion at -18, the P_1 gal promoter has become independent of cAMP-CRP, whereas P_2 is inactivated. A fourth mutation abolishes P_1 activity and diminishes P_2 activity. Each mutant has been characterized by DNA sequencing, in vitro transcription, CRP binding by DNAse footprinting, and in vivo enzyme measurements. Analysis of these mutants has led us to formulate a new model for CRP action.

Significance for Cancer Research and the Program of the Institute: Collagen is a major differentiation product of normal fibroblasts. Src type proteins, which are responsible for making cells oncogenic and phorbol esters, which have similar pleiotropic effects in cells cause a severe reduction of collagen synthesis. Our data indicate that this control, although indirect, is mediated by a transcriptional control mechanism. Understanding how the collagen gene is regulated should give us insights in the processes of differentiation and its alterations in transformation.

Proposed Course: (1) Study the regulation of the α_2 collagen gene by in vitro transcription using first crude extracts, then more purified components.

(2) Construct recombinants in which the promoter of the α_2 collagen gene controls the gene for an enzyme-like chloramphenicol acetylase or GPRTase bacterial genes which are capable of being expressed in animal cells. Transfect normal and transformed animal cells with such recombinants and study the expression of the collagen promoter.

(3) Obtain mutants in the α_2 collagen promoter by site specific mutagenesis; examine such mutants in vitro and in vivo after transfection.

(4) Isolate other collagen genes which appear to be differentiation products of other cell types (and which are also regulated by $p60^{src}$).

(5) Isolate one or more human collagen genes and examine the nature of the defects in some genetically transmitted diseases affecting one of the collagen genes.

(6) Obtain additional promoter mutants in the galactose operon by site-specific mutagenesis and analyze them by a variety of functional tests.

Publications:

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Sobel, M.E., Yamamoto, T., de Crombrugghe, B., and Pastan, I. Regulation of procollagen mRNA levels in Rous sarcoma virus-transformed chick embryo fibroblasts. Biochemistry, in press, 1981.

Avvedimento, E., Yamada, Y., Lovelace, E., de Crombrugghe, B., Pastan, I. Decrease in the levels of nuclear RNA precursors for α_2-collagen in Rous sarcoma virus transformed fibroblasts. Nucleic Acids Research, in press, 1981.

Vogeli, G., Ohkubo, H., Avvedimento, V.E., Yamada, Y., Mudryj, M., Pastan, I., and de Crombrugghe, B. A repetitive structure in the chick α_2-collagen gene. Cold Spring Harbor Symposia on Quantitative Biology, in press, 1981.

Pastan, I., Willingham, M., de Crombrugghe, B., and Gottesman, M.M. Aging and Cancer: Cyclic AMP and altered gene activity. Proceedings International Symposium on Aging, in press, 1981.

O'Neill, M.C., Amass, K., and de Crombrugghe, B. Molecular model of the DNA interaction site for the cyclic AMP receptor protein. Proc. Natl. Acad. Sci. USA, in press, 1981.

Vogeli, G., Ohkubo, H., Sobel, M., Yamada, Y., Pastan, I., and de Crombrugghe, B. Structure of the promoter for the chick α_2 collagen gene. Proc. Natl. Acad. Sci. USA, in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08702-20 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Endocytosis in the Thyroid Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Seymour H. Wollman Chief, Cell Organization Section, LMB, NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Cell Organization Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The typical thyroid epithelial cell can take in colloid from the follicular lumen by macropinocytosis. It can also phagocytose red blood cells. We propose to study the mechanism of these processes by electron microscopy, histochemistry, and related techniques.

PHS-6040
(Rev. 2-81)

Serial No. Z01 CB 08702-20 LMB
1. Laboratory of Molecular Biology
2. Cell Organization Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Endocytosis in the Thyroid Gland

Previous Serial Number: Z01 CB 08702-19 LMB

Principal Investigator: Seymour H. Wollman

Man Years: October 1, 1980 through September 30, 1981
Total: 0.0
Professional: 0.0
Other: 0.0

Project Description:

Objectives: To determine mechanism of thyroid hormone release and, more generally, endocytotic properties of typical thyroid epithelium.

Methods: Project was not pursued this year.

Significance for Cancer Research and the Program of the Institute: This project fits most clearly under Objective 3, Approach 5, having to do with properties of the cell surface.

Proposed Course: To be continued.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08703-29 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Thyroid Hormone Synthesis and Storage

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Seymour H. Wollman Chief, Cell Organization Section LMB, NCI

COOPERATING UNITS (if any)
R. Ekholm Department of Anatomy, University of Goteborg, Sweden

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Cell Organization Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has as its objectives to learn about: (1) The mechanism by which the thyroid gland maintains a concentration of iodide elevated above that of the blood, and (2) The mechanism by which the thyroid gland forms and accumulates protein-bound iodine.

The work is largely based upon in vivo studies and makes use of autoradiography, cytochemistry at ultrastructural level, kinetic studies, and inhibitors of steps in the synthesis of thyroid hormones.

PHS-6040
(Rev. 2-81)

Serial No. Z01 CB 08703-29 LMB
1. Laboratory of Molecular Biology
2. Cell Organization Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project: Thyroid Hormone Synthesis and Storage

Previous Serial Number: Z01 CB 08703-28 LMB

Principal Investigator: Seymour H. Wollman

Man Years: October 1, 1980 through September 30, 1981
Total: 0.0
Professional: 0.0
Other: 0.0

Project Description:

Objectives: To learn about the mechanism of synthesis and storage of thyroid hormones.

Methods: Not worked on this year.

Proposed Course: To be continued.

Significance for Cancer Research and the Program of the Institute: Some of the work reported here may best be put into Objective 3, Approach 5 because they give information on properties of cell surface and cell membranes.

Publications:

Wollman, S.H. and Ekholm R. Site of iodination in hyperplastic thyroid glands deduced from autoradiographs. Endocrinology 108: 2082-2085, 1981

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08704-29 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Study of Transplantable Thyroid Tumors, Thyroid Growth and Involution

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Seymour H. Wollman	Chief, Cell Organization Section	LMB, NCI
Others:	Osamu Tachiwaki,	Visiting Fellow	LMB, NCI
	Corrado Garbi	Visiting Fellow	LMB, NCI
	Staffan Smeds	Visiting Scientist	LMB, NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Cell Organization Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To study the thyroid growth and the production and properties of transplantable thyroid tumors in the rat: changes occurring during growth, hyperplasia and involution, growth and properties of thyroid cells and thyroid tumor cells in culture.

We are also studying a variety of types of cells just recently recognized to be present in the normal thyroid gland and which may be the cells of origin of some of the different histologic types of thyroid tumors which have been observed in the past.

PHS-6040
(Rev. 2-81)

Serial No. Z01 CB 08704-29 LMB
1. Laboratory of Molecular Biology
2. Cell Organization Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Production and Properties of Transplantable Thyroid Tumors, thyroid growth and involution

Previous Serial No.: Z01 CB 08704-28 LMB

Principal Investigator: Seymour H. Wollman

Other Investigators: Osamu Tachiwaki, Corrado Garbi, Staffan Smeds

Man Years: October 1, 1980 through September 30, 1981

Total	5.0
Professional	4.0
Other:	1.0

Project Description:

Objectives: To study thyroid growth and the production and properties of transplantable thyroid tumors in the rat: changes occurring in the thyroid during growth, hyperplasia and involution, factors controlling thyroid differentiation.

Objectives at present: To study thyroid growth and involution of hyperplastic thyroid. We are also studying the properties of primary cultures especially of the thyroid epithelial cells arranged in follicles in suspension.

Methods:

Hyperplasia: Hyperplasia was produced in rat thyroid gland by feeding the goitrogen, thiouracil. Various details of the nature of the growth process were examined by light and electron microscopy.

Involution: (With Herveg and Tachiwaki) Involution of the hyperplastic thyroid gland was induced by removing the diet containing a goitrogen and feeding the rats a diet with high iodine content.

Tissue Culture: Thyroid gland was dissociated by collagenase treatment. Small clusters of epithelial cells were isolated from the dissociated tissue by centrifugation followed by filtration through fine nylon mesh. Clusters were cultured in suspension and their properties were examined by electron microscopy.

Major Findings: Tissue culture (with C. Garbi):

1. Properties of thyroid epithelial in inverted follicles: In inverted thyroid follicles in suspension culture the epithelial cells are stretched. They exhibit some of the same features as stretched fibroblasts in monolayer culture, such as stress fibers and loss of microvilli. These features are related to the stretching of cells and not to attachment.

2. Stabilization of follicles in suspension culture: Follicles in suspension culture invert in 5% calf serum. They can be stabilized so that the epithelium has normal polarity by the addition of acid soluble collagen to the medium.

Mitosis in thyroid arteries and veins (with S. Smeds): During the induction of thyroid hyperplasia the thyroid arteries and veins enlarge. This is in part, due to multiplication of cells in the vessels, endothelial cells responding first. The time course of tritiated thymidine labeling is different for different veins and is later for more distant parts of the same vein. The arterial endothelial cells are also labeled. These results indicate a complex regulatory mechanism.

Change in thyroid fat pads during the induction of thyroid hyperplasia and involution (with S. Smeds): During the development of thyroid hyperplasia induced by the feeding of thiouracil in a low iodine diet the brown fat pads at the poles of thyroid lobes undergo lypolysis. There is simultaneous gross enlargement of blood vessels in the fat pads. Within one week after the induction of involution, fat reaccumulates and the blood vessels return to normal size.

Dense cell fragments are found in the thyroid follicular lumen during the involution of the hyperplastic thyroid (with O. Tachiwaki): The dense fragments occurring in the follicular lamen are fragments of the thyroid epithelial cells and are not defined from red blood cells in the lumen. This was shown by histochemical test for peroxidase under conditions where red blood cells stain but epithelial cell cytoplasm does not stain.

Significance for Cancer Research and the Program of the Institute: Enables us to understand more about the organization of cells in normal tissues.

Proposed Course: I plan to continue the above studies.

Publications

Nitsch, L. and Wollman, S.H. TSH preparations are mitogenic for thyroid epithelial cells in follicles in suspension culture. Proc. Natl. Acad. Sci. USA, 77: 2743-2747, 1980.

Nitsch, L. and Wollman, S.H. Ultrastructure of intermediate stages in polarity reversal of thyroid epithelium in follicles in suspension culture. J. Cell Biol. 86: 875-880, 1980.

Ericson, L.E. and Wollman, S.H. Increase in the rough Endoplasmic Reticulum in capillary cells and pericytes in hyperplastic rat thyroid glands. Endocrinology 107: 732-737, 1980.

Ericson, L.E. and Wollman, S.H. Ultrastructural aspects of capillary fusion during the development of thyroid hyperplasia. J. Ultrastructure Res. 72: 300-315, 1980.

Zeligs, J. and Wollman, S.H. Ultrastructure of cytokinesis in blood capillary endothelial cells in thyroid gland in vivo. J. Ultrastructure Res. in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08705-05 LMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic and Biochemical Analysis of Cell Behavior

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael M. Gottesman	Chief, Molecular Cell Genetics Section	LMB NCI
Others:	Jose Castano	Visiting Fellow	LMB NCI
	Jacob Hochman	Visiting Scientist	LMB NCI
	Toolsee Singh	Guest Worker	LMB NCI
	Charles Roth	Guest Worker	LMB NCI
	Irene Abraham	Expert	LMB NCI
	George Vlahakis	Research Biologist	LMB NCI
	Margaret Chapman	Research Biologist	LMB NCI
	Clark McClurkin	Co-Step	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	6.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are utilizing the Chinese Hamster Ovary (CHO) fibroblast grown in vitro to study the genetics and biochemistry of some aspects of the behavior of cultured cells. Our work has emphasized morphology and its relationship to growth control, response to cyclic AMP, the role of cell surface antigens, receptors and internalization of their ligands in cell behavior, and the mechanism of cell transformation by tumor viruses. We have isolated a variety of different mutants with altered microtubules, abnormal morphology, or abnormal response to cyclic AMP and many of these have been characterized as to their biochemical defects. We have isolated cell mutants resistant to transformation by the product of the src gene of Rous sarcoma virus. We are establishing general procedures for the isolation of mutants unable to internalize specific ligands. These mutants are analyzed genetically using the techniques of somatic cell hybridization, gene cloning and gene transfer, and biochemically by classical enzymology, immunology, affinity labelling techniques and two-dimensional electrophoresis.

PHS-6040
(Rev. 2-81)

Serial No. Z01 CB 08705-05 LMB
1. Laboratory of Molecular Biology
2. Molecular Cell Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Genetic and Biochemical Analysis of Cell Behavior

Previous Serial No: Z01 CB 08705-04 LMB

Principal Investigator: Michael M. Gottesman

Other Investigators: Jose Castano, Jacob Hochman, Toolsee Singh, Charles Roth, Irene Abraham, George Vlahakis, Margaret Chapman, Clark McClurkin

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	6.5
Professional:	6.5
Other:	0.0

Project Description:

Objectives: To determine by genetic manipulation of CHO cells the cell proteins and processes needed to maintain the cytoskeleton, response to cAMP, transformation by Rous sarcoma virus, and internalization of ligands.

Methods: Cell culture; virus infections; isolation of cell behavior mutants; karyotyping and G-binding; somatic cell hybridization; gene transfer using DNA and chromosomes; recombinant DNA techniques; immunologic techniques including immunoprecipitation, immunolocalization and use of monoclonal antibodies; one and two-dimensional electrophoretic and chromatographic analysis of DNA, RNA and proteins: enzymologic analyses.

Major Findings: (1) We have isolated multiple classes of mutants comprising more than 25 independent mutants which are resistant to the morphologic, transport, and growth inhibitory effects of increased levels of cyclic AMP. Seven of these independent mutants have been analyzed in detail. The major class is composed of mutants with altered cAMP dependent protein kinases indicating that these effects of cAMP in CHO fibroblasts are mediated through protein kinase. One of these mutants has a defect in the catalytic subunit of cAMP dependent protein kinase, and another has a defective Type I regulatory subunit. Some mutants lack type II protein kinase and some lack type I indicating that both of these enzymes are essential for all known cAMP effects in

CHO cells. One mutant appears to have normal protein kinase and may be defective in a subsequent step in cAMP action.

(2) We have analyzed the cAMP-dependent phosphorylations in intact wild-type and mutant CHO cells and in cell lysates. The major cAMP-stimulated phosphorylation in intact cells involves a protein of M_r approx. 52,000. Other phosphoproteins are also substrates in vivo for cAMP dependent protein kinase (e.g. intermediate filament protein) as detected by an alteration in the ^{32}P tryptic peptide fingerprints. In vitro, the major substrates have molecular weights of 17,000, 26,000, 34,000 and 300,000. The in vitro phosphorylations are all altered or blocked in the mutants with abnormal cAMP-dependent protein kinase activity, but the in vivo phosphorylations are only blocked in mutants lacking type II kinase.

(3) We have found that treatment of CHO cells with dibutyryl cAMP or cholera toxin results in a 3-5 fold increase in 8-azido-^{32}P-cAMP photoaffinity labeling of free type I regulatory subunit (RI) of cAMP dependent protein kinase. ^{35}S-met labeling studies demonstrate 2 isoelectric species of RI displayed on 2D gels. The molecular basis of the increased labeling of RI has been analyzed by purification of the mRNA species coding for these two RI forms.

(4) We have isolated several independent CHO mutants resistant to the anti-mitotic drugs colcemid, griseofulvin and taxol. By two-dimensional electrophoretic analysis, mutants resistant to colcemid and griseofulvin have been shown to contain an altered beta-tubulin and one resistant to taxol has an altered alpha tubulin. These proteins are the two major components of the microtubular system of the CHO cytoskeleton. Most of these mutants are temperature-sensitive for growth, and their defect at the non-permissive temperature is due to a failure of normal cell division. A class of temperature-resistant revertants of both alpha and beta-tubulin mutants has lost the mutant tubulin and become drug sensitive, proving that the temperature sensitivity and drug-resistance of these mutants are all related to their altered alpha or beta tubulin. The molecular basis of these alpha and beta-tubulin mutations has been analyzed using cloned alpha and beta-tubulin cDNAs.

(5) We have characterized the intermediate (10nm) filament system of CHO cells (another component of the cytoskeleton) and compared it to other cell types. As opposed to BHK or mouse epidermal cells, these filaments in CHO cells are composed of a single 55,000 M_r Triton-insoluble protein. Using homogeneous 55K protein, it is possible to reconstitute 10 nm filaments in vitro. The solubility properties of this protein and of heteropolymers formed with other intermediate filament components suggest that heteropolymers can be formed in vivo in cell types containing more than one intermediate filament protein type.

(6) We have successfully transformed a non-tumorigenic, flat revertant of our parent CHO cell line using Rous sarcoma virus and demonstrated

the presence of the src gene product in these cells. In this system, RSV transformation renders cells resistant to growth inhibition by cAMP without affecting protein kinase activity. We have isolated a large series of phenotypically non-transformed cell lines from a number of these RSV transformants and have characterized these. Preliminary evidence suggests that most of the revertants have lost the virus; others appear to have virus, but the expression of their transforming function is altered, either because src is altered or reduced in amount or because the host cell is unable to respond to a normal src product. Using ^{32}P - labeling of intact CHO cells and 2D gel analysis, we have identified several likely candidates as substrates for the protein kinase activity associated with src.

(7) As part of the development of techniques for the genetic analysis of CHO cells, we have successfully transformed these cells with DNA containing the cloned Herpes Simplex Virus thymidine kinase gene, a cloned E. coli xanthine-guanine phosphoribosyl transferase gene, and tk+ CHO whole chromosomal DNA. These results suggest that it will be possible to use DNA transfer in linkage analysis of CHO mutations, to study gene regulation in mutant cell lines and as a means of direct cloning of genes by plasmid rescue techniques.

Significance for Cancer Research and the Program of the Institute: CHO cells will cause tumors in appropriate hosts. The identification of mutant CHO cells with specific defects in cell surface functions and in regulation of cellular growth and morphology will enable us to determine whether any of these functions are needed for tumor formation. Once this information is obtained, specific therapy aimed at neutralizing those cell surface or cytoskeletal functions required for tumor formation can be devised.

Objective 3, Approach 3.5

Proposed Course: To isolate many classes of mutants with abnormal cell behavior and determine specific protein alterations in these mutants. To determine the molecular basis for mutants already isolated and to use recombinant DNA technology to molecularly clone the components of the cAMP dependent protein kinase system.

Publications:

Cabral, F., Willingham, M.C., and Gottesman, M.M. Ultrastructural localization to 10 nm filaments of an insoluble 58K protein in cultured fibroblasts. J. Histochem. Cytochem., 28: 653-662, 1980.

Cabral, F., Sobel, M.E. and Gottesman, M.M. CHO mutants resistant to colchicine, colcemid or griseofulvin have an altered beta-tubulin. Cell 20: 29-36, 1980.

Milhaud, P.G., Davies, P.J.A., Pastan, I., and Gottesman, M.M. Regulation of transglutaminase activity in Chinese hamster ovary cells. Biochem. Biophys. Acta 630: 476-484, 1980.

Milhaud, P., Yamada, K.M. and Gottesman, M.M. Sodium butyrate affects expression of fibronectin in CHO cells: Specific increase in antibody-complement-mediated cytotoxicity. J. Cell Physiol. 104: 163-170, 1980.

LeCam, A., Gottesman, M.M. and Pastan, I. Mechanism of cyclic AMP effect on nutrient transport in Chinese hamster ovary cells: A genetic approach. J. Biol. Chem. 255: 8103-8108, 1980.

Gottesman, M.M. Genetic approaches to cyclic AMP effects in cultured mammalian cells. Cell 22: 329-330, 1980.

Singh, T.J., Roth, C., Gottesman, M.M. and Pastan, I. Characterization of cyclic AMP resistant Chinese hamster ovary cell mutants lacking type I protein kinase. J. Biol. Chem. 256: 926-932, 1981.

LeCam, A., Nicolas, J.C., Singh, T.J., Cabral, F., Pastan, I. and Gottesman, M.M. Cyclic AMP-dependent phosphorylation in intact cells and in cell-free extracts from Chinese hamster ovary cells. Studies with wild-type and cyclic AMP-resistant mutants. J. Biol. Chem. 256: 933-941, 1981.

Cabral, F., Gottesman, M.M., Zimmerman, S.B. and Steinert, P.M. Intermediate filaments from Chinese hamster ovary cells contain single protein: Comparison with more complex systems from baby hamster kidney and mouse epidermal cells J. Biol. Chem. 256: 1428-1431, 1981.

LeCam, A. and Gottesman, M. M. Glycogen synthesase activity in Chinese hamster ovary cells. Studies with wild-type and mutant cells defective in cyclic AMP-dependent protein kinase. B.B.A., in press, 1981.

Gottesman, M. M. and Rabin, M. S. "High frequency loss of adenosine kinase activity in Chinese hamster ovary cells, in Intact Human Cell Lines in Study of Inborn Metabolic Diseases (NIAMDD Monograph), in press, 1981.

Pastan, I. H., Willingham, M., de Crombrugghe, B., and Gottesman, M. M. Aging and cancer: Cyclic AMP and altered gene activity, in Proceedings of the International Symposuim on Aging and Cancer in press, 1981.

Gottesman, M. M., Singh, T., LeCam, A., Roth, C., Nicolas, J.-C. Cabral, F., and Pastan, I. Cyclic AMP-dependent phosphorylations in cultured fibroblasts: A genetic approach, in Cold Spring Harbor Conferences on Cell Proliferation, in press, 1981.

Cabral, F., Abraham, I., and Gottesman, M. M. Isolation of a taxol-resistant Chinese hamster ovary cell mutant with an alteration in alpha-tubulin. Proc. Natl. Acad. Sci., in press, 1981.

Steinert, P. M., Idler, W. W., Cabral, F., Gottesman, M. M., and Goldman, R. D. In vitro assembly of homopolymer and copolymer filaments from intermediate filament subunits of muscle and fibroblasts cells. Proc. Natl. Acad. Sci., in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08706-10 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Mammary Carcinogenesis: MMTV - Cell Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Gilbert H. Smith, Research Biologist LMB, NCI

COOPERATING UNITS (if any)
LVC, DCCP, NCI
Department of Cell Biology, Baylor University
Biological Carcinogenesis Branch, Frederick Cancer Research Center

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Cell Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

C3H/Sm mice have lost the exogenous milk-borne mammary tumor virus characteristic of the C3H strain and have a very low (1.5%) incidence of spontaneous mammary tumors. They are highly susceptible to mammary carcinogenesis by either chemical carcinogens or infection with the milk-borne virus. We have analyzed the MMTV proviral DNA content of normal tissues and of spontaneous, virus-induced, and chemically-induced mammary tumors by restriction endonuclease digestion and Southern blot analysis. While the results clearly show additional MMTV sequences in the virus-induced tumor not present in liver DNA, none of the spontaneous or chemically-induced tumors can be shown to contain newly acquired either exogenous or amplified endogenous MMTV sequences. Examination of the susceptibility of restriction sites to isoschizmers of the restriction endonucleases sensitive to the presence of methyl groups provided evidence that hypomethylation of some of the MMTV proviral sequences occur in the spontaneous and chemically induced C3H/StWi tumors. These sites are hypermethylated in normal C3H/StWi tissues suggesting that these genes are actively transcribed in the mammary tumors.

Serial No. Z01 CB 08706-10 LMB
1. Laboratory of Molecular Biology
2. Molecular Cell Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Mammary Carcinogenesis: MMTV - Cell Interaction

Previous Serial No: Z01 CB 08706-09 LMB

Principal Investigator: Gilbert H. Smith, Research Biologist, Laboratory of Molecular Biology, DCBD, NCI

Other Investigators: Dale E. Graham, Cancer Expert

Cooperating Units: LPP, LB, DCBD, NCI
Department of Cell Biology, Baylor College of Medicine
Biological Carcinogenesis Branch, Frederick Cancer Research Center
LVC, DCCP, NCI

Man Years: October 1, 1980 through September 30, 1981

Total:	3.0
Professional:	2.0
Other:	1.0

Project Description:

Objectives: The aim of the project is to elucidate the cell-virus-provirus interactions in the mouse mammary gland leading to epithelial cell hyperplasia and eventually to neoplasia. Multiple factors, including virus, chemicals, hormonal stimulation of the gland and genetic susceptibility of the individual, have been identified as playing important roles in the development of mammary cancer. Therefore our approach includes a multidisciplinary analysis of the intracellular events associated with mammary gland differentiation and development and the role of the mammary tumor virus and chemical carcinogens in these normal events and those accompanying preneoplasia and neoplasia in the mammary epithelial tissue. We have initiated investigations into the role of chemical carcinogens in mammary cancer to determine whether they operate synergestically or independently of the tumor virus and whether chemicals have different targets within the tissue than the virus. To this end we are evaluating a new mouse model for experimental breast cancer, which gives promise of greater relevancy to the human condition. Special emphasis will be given to the physiological state of the epithelial tissue in relation to its response to the carcinogenic agent.

Major Findings: In collaboration with Dr. B. Vonderhaar (LPP, DCBD),

confirmed that hormone mediated functional differentiation in virgin mouse mammary epithelial explants requires a DNA-synthesis-linked event independent of cytokinesis. Thus mitosis is not the critical event in this tissue during functional differentiation.

In studies of chemical carcinogenesis of the mammary gland with Dr. D. Medina, Baylor College of Medicine, the rate and nature of neoplastic transformation of the epithelium was found to be unaffected by the presence of highly oncogenic retrovirus (MMTV) in the mammary tissue. This provided evidence that chemical and viral oncogens follow separate pathways to malignant transformation in mammary epithelium. With the cooperation of Dr. Medina, Baylor College of Medicine and Dr. Y.A. Teramoto, DCCP, we discovered that no qualitative or quantitative alteration in MMTV proviral gene expression accompanied tumorigenesis in the mammary gland in C3H/Sm mice in the presence or absence of chemical carcinogens (DMBA or Urethan) and/or excess hormone stimulation (pituitary isografts). This supports the conclusion that MMTV proviral expression is not a requirement for tumorigenesis in mouse mammary gland.

C3H/Sm mice were shown to be partially defective for the expression of MMTV proviral genes. Although MMTV RNA was transcribed which was representative of the complete viral genome, only 30-40% of these sequences were present in the cytoplasm. This characteristic of C3H/Sm mice has been shown to be heritable and dominant leading to the diminition of mammary tumor incidence in hybrid mice. This characteristic of C3H/Sm mice is under further study.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 3. The mouse mammary tumor virus problem is a relevant experimental model for understanding human mammary neoplasia. The project is designed to provide a scientific basis to further our understanding of intracellular events during normal development of the mammary gland as well as during malignant transformation.

Proposed Course: To specify the tumorigenic influence of the mammary tumor virus, its relationship to normal differentiation and to the cellular response during chemical carcinogenesis, further to clarify the regulatory events responsible for the expression of endogenous retrovirus genes and their role in mammary tumorigenesis.

Publications:

Smith, G.H., Mirski, M., and Arthur, L.O. DNA binding and unwinding activities associated with intracytoplasmic A particles isolated from mammary tumors. J. Gen Virol., 49: 263-272, 1980.

Smith, G.H., Arthur, L.O. and Medina, D. Evidence of separate pathways for viral and chemical carcinogenesis in C3H/StWi mouse mammary glands. Int. J. Cancer 26: 373-379, 1980.

Smith, G.H., Teramoto, Y.A. and Medina, D. Hormones, chemicals and proviral gene expression as contributing factors during mammary carcinogenesis in C3H/StWi mice. Int. J. Cancer 27: 81-86, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08707-08 LMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Relationship of Genetic and Non-genetic Factors in Mouse Mammary Tumorigenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George Vlahakis, Research Biologist	LMB,NCI
Other:	Bernard Sass, Research Pathologist	CGT,NCI

COOPERATING UNITS (if any)
Carcinogenesis Testing Program, DCCP, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Cell Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.25	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Collaborative work continues into the relationship of genetic and non-genetic factors in spontaneous mouse tumorigenesis. Interest is in the different lines of naturally occurring mammary tumor virus (MTV), and in the genetically variable inbred mouse strains. Interest also is in the manner of transmission of the various MTV's, i.e. whether by female or equally well by either parent. Finally, strains of mice that develop hormone responsive mammary gland tumor and hyperplastic alveolar nodules from which malignant mammary tumor arise are also of interest for histogenesis studies.

PHS-6040
(Rev. 2-81)

Serial No. Z01 CB 08707-08 LMB
1. Laboratory of Molecular Biology
2. Molecular Cell Genetics Section
3. Bethesda, Maryland 20205

PHS-NIH
Individual Project Report
October 1, 1980 to September 30, 1981

Project Title: The Relationship of Genetic and Non-genetic Factors in Mouse Mammary Tumorigenesis

Previous Serial No.: Z01 CB 08707-07 LMB

Principal Investigator: George Vlahakis, Research Biologist, Laboratory of Molecular Biology, DCBD, NCI

Other Investigators: Bernard Sass, Research Pathologist, CGT, NCI

Cooperating Units: Carcinogenesis Testing Program, DCCP, NCI

Man Years: October 1, 1980 to September 30, 1981

Total: .25
Professional: .25
Other:

Project Description: Objective 1, Approach 4

Collaborative studies with other investigators both here and outside the NIH are continuing again this year.

In collaboration with Melvin D. Reuber of the Frederick Cancer Research Center, a survey has been completed and accepted for publication reporting on the spontaneous occurrence of uterine tumors arising in our laboratory strains of female mice. The paper will appear in the Journal of Gerontology. We believe that a histologic description of these lesions will be useful to those investigators working with mice and possibly other rodents. This year we are planning to submit another paper for publication describing more thoroughly the development of leiomyosarcoma, one of the spontaneous malignant mouse tumors in our survey.

A manuscript with Gerald L. Princler and K. Robert McIntire of the Laboratory of Immunodiagnosis here at NIH is now being revised for publication in the European Journal of Cancer. It deals with the dynamics of serum alpha-fetoprotein occurring in the high spontaneous hepatoma mouse strain C3H-A^{vy}fB.

A paper on the histogenesis and biology of mammary gland lesions occurring spontaneously in female mice is now in preparation with Bernard Sass of the Registry of Experimental Cancer, Division of Cancer Cause and Prevention. One of the precursor lesions we are studying is the hyperplastic alveolar nodule from which most mammary tumors arise in females carrying mammary tumor virus (MTV). Another precursor mammary gland lesion of interest to us is the plaque which occurs in several of the high mammary tumor mouse strains not native to the United States. We are also interested in the pregnancy dependent mammary tumor observed in the European strain GR. These tumors normally regress at parturition but reappear in subsequent pregnancies and eventually become malignant. One morphologically distinct mammary tumor in GR classified as pale cell carcinoma is of particular interest. This lesion apparently is associated with the line of MTV present in GR.

Significance to Cancer Research and the Program of the Institute: The occurrence of spontaneous mammary gland tumors in laboratory mouse strains depends on the interplay of several factors: host genotype, mammary tumor virus, hormones, environment. Mouse mammary tumorigenesis with the variable tumor strains needs to be continued so that eventually mechanisms of action between the various factors will be understood and might, therefore, provide better insight into the disease process in humans.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08709-06 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Relationship of Carbohydrate and NAD^+ Metabolism to Cell Growth

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George S. Johnson, Research Chemist	LMB, NCI
Others:	Carolyn R. Adler, Guest Worker (summer worker)	LMB, NCI
	Narimichi Kimura, Visiting Fellow	LMB, NCI
	Nobuko Kimura, Visiting Associate	LMB, NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Cell Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	3.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cyclic AMP accumulation following addition of adenylate cyclase activators is augmented in cultured normal rat kidney (NRK) cells treated with 2-pyridine carboxylic acid. This activation is due to increases in GTP-dependent adenylate cyclase activities, measured in purified plasma membranes. Cyclic nucleotide phosphodiesterase activity is unaffected.

NAD and 1-methylnicotinamide were analyzed in several clones of normal and transformed NRK cells. Intracellular levels of both metabolites did not correlate with transformation or growth rate. Benzamide and its derivatives, known inhibitors of poly (ADP-ribose) synthetase, were demonstrated to inhibit also the enzyme, nicotinamide methyltransferase.

PHS-6040
(Rev. 2-81)

Serial No. Z01 CB 08709-06 LMB
1. Laboratory of Molecular Biology
2. Molecular Cell Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Relationship of Carbohydrate and NAD^+ Metabolism to Cell Growth

Previous Serial Number: Z01 CB 08709-05 LMB

Principal Investigator: George S. Johnson

Other Investigators: Narimichi Kimura, Nobuko Kimura and Carolyn R. Adler

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981
Total: 3.0
Professional: 3.0
Other: 0.3 (summer help)

Project Description:

Objectives: Nicotinamide adenine dinucleotide (NAD) is known to undergo two major types of reactions in mammalian cells, reduction to NADH and cleavage at the nicotinamide-ribose linkage to form nicotinamide and adenosine diphosphate ribose. ADP-ribose can be covalently attached to proteins or it can polymerize to form poly (ADP-ribose). Functions of NAD is present in the cell at a concentration of about 0.1-0.3 mM, and since the half time for degradation of total cellular NAD at this cleavage site is just 1-2 hours, this reaction is major cellular activity.

The object of this project is to understand possible involvements of NAD^+ in cellular functions. Emphasis will be placed on growth regulation, macromolecular synthesis, and cyclic AMP metabolism.

Methods: Standard culture techniques will be used to grow cells. Thin layer chromatography and fluorescent analysis will be used to analyze metabolic components. Standard biochemical techniques will be used to measure cyclic AMP, adenylate cyclase activity, and RNA metabolism.

Major Findings: NAD and nicotinamide metabolism: NAD and 1-methyl-nicotinamide are the major nictotinamide metabolites in cultured normal rat kidney (NRK) cells. Alterations in metabolism of both compounds have been reported in malignant tissues indicating a possible importance in cancer. Their levels were therefore measured in several clones of untransformed NRK cells and in NRK cells transformed by different tumor viruses. The intracellular levels of these metabolites were not altered consistently by transformation and did not correlate with doubling time in the various cell lines. Attempts were made to determine possible physiological roles for 1-methylnicotinamide.

As mentioned above, its intracellular levels did not correlate with growth rate or transformation. No evidence was obtained that its synthesis is involved with maintenance of nicotinamide or S-adenosylmethionine levels. Thus the biological function for this metabolite remains unknown.

Benzamide and its derivatives have been reported to inhibit poly (ADP-ribose) synthetase activity in many cell systems. We found that these drugs also inhibited nicotinamide methyltransferase.

Cyclic AMP metabolism: The ability of adenylate cyclase agonists to increase intracellular levels of cyclic AMP was augmented in cells treated by various means. Some of these treatments are known to alter NAD metabolism in other cell systems. Treatment with 2-pyridine carboxylic acid (picolinic acid) gave largest and most consistent results. The increase with this agent was due to a potentiation of GTP-dependent adenylate cyclase activities. Cyclic nucleotide phosphodiesterase activity was unaffected. Studies are underway to determine the molecular alterations in adenylate cyclase. An augmented adenylate cyclase activity was retained in sucrose gradient-purified plasma membranes demonstrating that cytoplasmic factors were not essential. Also, membrane proteins synthesized in a short pulse with a radioactive amino acid probe in control and treated cells were almost identical in two dimensional gels showing that effects of picolinic acid treatment were not widespread but rather quite specific for adenylate cyclase.

Significance for Cancer Research and the Program of the Institute: By understanding how growth and metabolism are regulated and by understanding differences in this regulation between normal and transformed cells we may learn to control the growth of certain cancer cells.

Proposed course: Adenylate cyclase will be analyzed in detail in purified membranes to determine the molecular basis for its augmented activity. Attempts will be made to determine how the various treatments perturb the cell in order to induce these changes in adenylate cyclase.

The enzymes poly (ADP-ribose) synthetase, NAD glygohydrolase, poly (ADP-ribose) glycohydrolase and nicotinamide methyltransferase will be analyzed in control and treated cells. Also, the ADP-ribosylation of specific nuclear and nucleolar proteins will be determined.

Publications:

Kidwell, W.R. Stone, P.R. and Johnson, G.S. Poly (ADP Ribose) Synthesis and chromatin structure. In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins. T. Sugimura and M. Smulson, Eds. Elsevier North-Holland, New York. pp. 73-84, 1980.

Johnson, G.S. Effects of pyridine derivatives on cultured cells: evidence for an involvement of ADP-ribosylation in growth regulation and cyclic AMP-metabolism. In: Novel ADP-Ribosylations of Regulatory Enzymes and proteins. T. Sugimura and M. Smulson, Eds. Elsevier North-Holland, New York pp. 295-302, 1980.

Johnson, G.S. Metabolism of NAD and N^1-methylnicotinamide metabolism in growing and growth-arrested cells. Eur. J. Biochem. 112: 635-641, 1980.

Constantini, M.G. and Johnson, G.S. Disproportionate accumulation of 18S and 28S ribosomal RNA in cultuted normal rat kidney cells treated with picolinic acid or 5-methylnicotinamide. Exp. Cell Res., in press, 1981.

Johnson, G.S., Chiang, P.K. 1-methylnicotinamide and NAD metabolism in normal and transformed normal rat kidney cells. Archives Biochem. Biophys., in press, 1981.

Johnson, G.S. Benzamide and its derivatives inhibit nicotinamide methylation as well as ADP-ribosylation. Biochemistry International, in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08710-06 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

DNA Replication and Recombination In Vitro.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Sue Wickner, Research Chemist LMB NCI

Other: Ariella Oppenheim LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. The molecular mechanisms involved in DNA replication are being studied. Attempts are being made to reproduce in vitro, the in vivo pathways of replication of bacteriophage λ DNA in extracts of λ infected bacteria and of viral SV40 DNA in extracts of viral infected or transformed animal cells. The present emphasis in both cases is to gain insight into the process of initiation of chromosome replication.

2. The molecular mechanisms involved in DNA recombination are also being studied. The reaction by which prophage λ is excised from the bacterial chromosome has been reconstituted with purified proteins in vitro. The interactions of these proteins with each other and DNA are being characterized.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08710-06 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: DNA Replication and Recombination In Vitro.

Previous Serial Number: ZO1 CB 08710-05 LMB

Principal Investigator: Sue Wickner

Other Investigator: Ariella Oppenheim

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	1.5
Professional:	1.5
Others:	0.0

Project Description:

Objective: To gain information about basic biochemical mechanisms involved in the process of DNA replication and recombination.

Methods Employed: 1. Biochemical techniques required for protein purification, enzyme assays, phage and plasmid DNA preparation, and nucleic acid sequencing.

2. Standard techniques for growing bacteria, phage, animal cells, and viruses.

Major Findings: 1. Initiation of λ DNA replication. λ replication in vivo requires two phage proteins, the O and P gene products, in addition to host proteins including dnaB, polC, dnaG, dnaN, dnaZ, dnaK, grpD and grpE gene products and RNA polymerase. In vivo experiments have shown that λO protein is required for initiation of replication of the λ chromosome. It has also been shown to interact with the λ origin of replication and the λP protein. I am now working on the isolation of λO protein from cells carrying a plasmid on which the O gene was cloned by K. Zahn in Dr. F. Blatner's laboratory. Future studies will include the characterization of the O protein and its binding to DNA and the λP protein. I have purified the λP protein as well as many of the E. coli proteins required for λ replication so it may be possible to reconstruct λ replication in vitro.

2. Recombination of λ. The excision of λ from the E. coli chromosome requires two phage functions, int and xis gene products, at least two host functions, himA and hip gene products, and the right and left attachment sites of prophage λ. The int protein and a host factor (composed of two polypeptides) have been purified and characterized in the λ integration reaction. Using the in vitro excision reaction developed by Dr. K. Abremski in Dr. S. Gottesman's laboratory, I have purified xis and the same host factor required for λ integration. Now I am studying the interaction of xis protein with int protein and with DNA containing λ attachment sites.

3. SV40 DNA replication. SV40 replication in vivo requires the viral A protein as well as host functions. Dr. A. Oppenheim and I are attempting to replicate exogenously added SV40 viral DNA in extracts prepared from SV40 infected and transformed cells. We can detect newly synthesized supercoiled SV40 DNA dependent on added SV40 DNA in extracts of infected cells. We are currently characterizing this reaction.

Significance for Cancer Research and the Program of the Institute: The central process of heredity and cell growth is the replication of the genetic material. Studies of this process in E. coli have been made possible by the availability of (1) mutants defective in DNA synthesis and (2) large amounts of bacteria necessary for biochemical studies. It is expected that the understanding that this work is generating about the process of DNA replication in E. coli will shed light on the nature of the same process in animal cells. Perhaps if normal replication were understood, we would be better able to study the abnormalities of cancer cells.

Proposed course: To continue studying biochemical mechanisms involved in DNA synthesis and recombination using purified proteins and defined DNA templates.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08711-05 LMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanism of Site-Specific Recombination in Bacteriophage Lambda

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Susan Gottesman, Research Chemist	LMB NCI
Others:	Patsy Trisler, Research Biologist	LMB NCI
	Kenneth Abremski, Guest Worker	LMB NCI
	Jeffrey Auerbach, Guest Worker	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been studying the biochemistry and genetics of lambda site-specific recombination. We have purified some of the proteins involved in excisive site-specific recombination of bacteriophage lambda. We are studying the role of the phage-coded function, Xis and Int, and host-coded functions in this recombination reaction. We are extending these studies to the in vitro recombination reaction. We are isolating mutations in Int and Xis which alter the interaction of these proteins during integration and excisive recombination.

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(Rev. 2-81)

Serial No. ZO1 CB 08711-05 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Mechanism of Site-Specific Recombination in Bacteriophage Lambda

Previous Serial Number: ZO1 CB 08711-04 LMB

Principal Investigator: Susan Gottesman

Other Investigators: Patsy Trisler, Kenneth Abremski, and Jeffrey Auerbach

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	0.8
Professional:	0.8
Other:	0.0

Project Description:

Objectives: Bacteriophage lambda inserts into and excises from the E. coli chromosome. This recombination occurs at a unique site in the viral and host chromosomes, and is promoted by several functions. We have previously demonstrated the excision reaction in vitro. Our object is to continue to study the mechanism of phage excision, by purifying and analyzing the components of the in vitro system, and to understand how the excision reaction differs from the forward, integration reaction at a mechanistic level.

Methods Employed: Standard microbial genetic and biochemical techniques.

Major Findings: (1) Using a plasmid, constructed in this laboratory, which overproduces Xis, a significant purification of Xis has been achieved. Recombination reactions can now be studied with totally purified components (Int, Xis, Host Factors, and DNA).

(2) Xis inhibits the integration reaction in vitro, but not in vivo. This difference may reflect: (a) inadequate gene dosage of Xis in vivo to inhibit, (b) inability of Xis, which is highly unstable in vivo but not in vitro, to inhibit due to its instability, or (3) some other difference in in vivo vs. in vitro conditions.

We have devised genetic selections for mutants which require that Xis inhibit integration in vivo. Characterization of the mutants should reveal something about the nature of the inhibition, and may help in biochemical studies of Xis by providing mutants which no longer are unstable for Xis activity. In addition, mutant selections have been specifically designed to isolate host or phage mutants which lead to stable Xis.

Significance for Cancer Research and the Programs of the Institute: Integration and excision of the bacterial virus lambda may serve as a model for understanding the integration and excision mechanisms of animal viruses.

Proposed Course: Purification of host factors involved in excision; identification of the role of Xis in site-specific recombination and its mechanism of interaction with Int and DNA; comparison of the roles of Xis and Him A. Isolation of mutants affecting Xis-Int interactions.

Publications:

Gottesman, S., and Abremski, K. The role of Him A and Xis in lambda site-specific recombination J. Mol. Biol. 138: 503-512, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08712-06 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ira Pastan, Chief, Laboratory of Molecular Biology	LMB NCI
	Mark Willingham, Head, Ultrastructural Cytochemistry Section	LMB NCI
Others:	Robert Dickson, Guest Worker	LMB NCI
	Richard Schlegel, Assistant Senior Surgeon	LMB NCI
	Alexander Levitski, Visiting Scientist	LMB NCI
	Harry Haigler, Guest Worker	LMB NCI
	Paul Dragsten	
	Jacob Robbins	

COOPERATING UNITS (if any)
Dept. Bioch. Hebrew, Univ., Jerusalem, Isreal
Lab. Theor. Biol. NCI, Clin. Endo. Branch, NIAMDD

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.9	2.9	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have identified a new organelle, the receptosome that carries ligands from the clathrin coated pits on the surface of fibroblasts to the Golgi-GERL apparatus and in some cases on to lysosomes. Ligands carried by this pathway include $\alpha_2$macroglobulin, epidermal growth factor, insulin, low density lipoprotein, β-galactosidase and some viruses.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08712-06 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior

Previous Serial Number: ZO1 CB 08712-05 LMB

Principal Investigators: Ira Pastan and Mark Willingham

Other Investigators: Robert Dickson, Richard Schlegel, Alexander Levitski, and Harry Haigler

Cooperating Units: Dept. Bioch. Hebrew, Univ., Jerusalem, Isreal
Lab. Theor. Biol. NCI, CEB, NIAMDD

Man Years: October 1, 1980 through September 30, 1981

Total:	3.9
Professional:	2.9
Other:	1.0

Project Description:

Objectives: To identify the membrane proteins that participate in cell adhesion, cell movement and regulate cell metabolism and growth and to determine if these are altered in malignant transformation.

Methods: Cell culture; preparation and analysis of membrane proteins and their effects on cell behavior; isolation of mutants with defective membrane proteins.

Major Findings: With M. Willingham and coworkers we have identified a new organelle the receptosome that carries ligands from coated pits in the plasma membrane to the cell interior. The receptosome prevents ligands from being directly transferred to lysosomes and instead transports them to the Golgi from which some ligands go on to lysosomes. Ligands that traverse this pathway are $\alpha_2$macroglobulin (α_2M), insulin, epidermal growth factor, triiodothryronine, low density lipoprotein, (with D. Via, A. Gotto and C. Smith), β-galactosidase (with G. Sahagian and E. Neufeld). In addition some viruses including vesicular stomatitis virus (VSV) and adenovirus use the coated pit receptosome pathway.

α_2M entry is inhibited by a variety of compounds that also inhibit the cellular enzyme transglutaminase. These include dansylcadaverine (DC), bacitracin (B) and BDONV (blocked diazoxonorvaline). These agents prevent clustering of α_2M-receptor complexes in coated pits. R. Dickson has shown these compounds also inhibit high affinity binding of α_2M to intact fibroblasts and fibroblast membranes suggesting that the high affinity binding is due to the clustering of the L-R complexes in coated pits and the low affinity binding is to binding to sites that are unclustered outside coated pits.

Not all ligands that enter cells by coated pits are equally sensitive to all classes of the inhibitors: α_2M, insulin and T_3 are sensitive to DCD and BDONV. EGF only responds to high concentrations of DC. LDL is very sensitive to DC but not B. The identity of the coated pit components responsible for ligand-receptor clustering is under investigation.

R. Schlegel has begun to look in detail at the mechanism of the entry of a virus, VSV and how this virus escapes from the receptosome into the cytoplasm.

Significance to Biomedical Research and the Program of the Institute: The invasive properties of tumor cells may be due to altered membrane proteins. Identification and isolation of the proteins may suggest new methods of cancer treatment. Objective 3, Approach 3.5.

Proposed Course: Identify how receptor-ligand complexes cluster and how the receptosome is formed and moves to Golgi.

Publications:

Pastan, I., Sobel, M., Adams, S., Yamada, K.M., Howard, B., Willingham, M., and de Crombrugghe, B. The use of transformed fibroblastic cells as a model for differentiation. In Differentiation and Development Tenth Miami Winter Symposium, eds. Ahmad, F., Schultz, J., Russell, T.R., and Werner, R. (Academic Press, N.Y.), Vol. 15, 1978, p. 49-54.

Davies, P.J.A., Wallach, D., Willingham, M., Pastan, I., and Lewis, M.S. Self-association of chicken gizzard filamin and heavy merofilamin. Biochemistry 19: 1366-1372, 1980.

Levitzki, A., Willingham, M., and Pastan, I. Evidence for participation of transglutaminase in receptor-mediated endocytosis. Proc. Natl. Acad. Sci. USA 77: 2706-2710, 1980.

Cheng, S., Maxfield, F.R., Robbins, J., Willingham, M.C., and Pastan, I.H. Receptor-mediated uptake of 3,3'5-triiodo-L-thyronine by cultured fibroblasts. Proc. Natl. Acad. Sci. USA 77: 3425-3429, 1980.

Willingham, M.C. and Pastan, I. The receptosome: An intermediate organelle of receptor-mediated endocytosis in cultured fibroblasts. Cell 21: 67-77, 1980.

Willingham, M.C., Maxfield, F.R., and Pastan, I. Receptor-mediated endocytosis of α_2-macroglobulin in cultured fibroblasts. J. Histochem. Cytochem. 28: 818-823, 1980.

Pastan, I.H. and Willingham, M.C. Receptor-mediated endocytosis of hormones in cultured cells. Annual Review of Physiology 43: 239-250, 1981.

Haigler, M.T., Willingham, M.C., and Pastan, I. Inhibitors of ^{125}I-epidermal growth factor internalization. Biochem. Biophys. Res. Commun. 94: 630-637, 1980.

Maxfield, F.R., Willingham, M.C., Pastan, I., Dragsten, P., and Cheng, S.-Y. Binding and mobility of the cell surface receptors for 3,3',5-triiodo-L-thyronine. Science 211: 63-65, 1981.

Maxfield, F.R., Willingham, M.C., Haigler, H.T., Dragsten, P., and Pastan, I. Binding, surface mobility, internalization and degradation of rhodamine labeled α_2-macroglobulin. Submitted Biochemistry, 1980.

Dickson, R.B., Willingham, M.C., and Pastan, I. Binding and internalization of ^{125}I-α_2 macroglobulin by cultured fibroblasts. J. Biol. Chem. 256: 3454-3459, 1981.

Keen, J., Willingham, M., and Pastan, I. Clathrin and coated vesicle proteins: Immunological characterization. J. Biol. Chem., 256: 2538-2544, 1981.

Dickson, R.B., Willingham, M.C., and Pastan, I. α_2-Macroglobulin adsorbed to colloidal gold: A new probe in the study of receptor-mediated endocytosis. J. Cell Biol. 89: 29-34, 1981.

Willingham, M.C., Haigler, H.T., Dickson, R.B., and Pastan, I. Receptor-mediated endocytosis in cultured cells: coated pits, receptosomes, and lysosomes. Submitted Intl. Cell Biol., 1980 (book).

Pastan, I.H. and Willingham, M.C. The internalization of insulin and other hormones by fibroblastic cells. Festshrift Symposium Honoring Dr. Rachmiel Levine, Eisenhower Medical Center, Rancho Mirage, CA, 1980.

Pastan, I., Haigler, H., Dickson, R., Cheng, S-Y., and Willingham, M. The role of the receptosome in receptor-mediated endocytosis. Proceedings of the Miami Winter Symposium on Cellular Responses to Molecular Modulators. Academic Press, 1981. Miami Winter Symposium held 1/12-16/81, Miami, Florida in press.

Dickson, R.B., Nicolas, J-C., Willingham, M.C., and Pastan, I. Internalization of α_2-macroglobulin in receptosomes: Studies with monovalent electron microscopic markers. Exp. Cell Res., in press, 1981.

Pastan, I., Willingham, M., de Crombrugghe, B. Aging and cancer: Cyclic AMP and altered gene activity. Submitted JNCI-Monograph, 1981.

Macchia, V., Caputo, G., Mandato, E., Rocino, A., Adhya, S., and Pastan, I. Guanylate cyclase activity in mutants of E. coli defective cyclase. Submitted J. Bact., 1981.

Willingham, M.C. and Pastan, I.H. The morphologic pathway of receptor-mediated endocytosis in cultured fibroblasts for Unilever 50th Anniversary Symposium Proceedings, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08714-04 LMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mode of Action of a Bacterial Function Involved in Cell Growth Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Susan Gottesman, Research Chemist	LMB NCI
	Patsy Trisler, Research Biologist	LMB NCI
	Max Gottesman, Chief, Biochemical Genetics Section	LMB NCI
	Saeko Mizusawa, Visiting Fellow	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

National Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.8	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The lon mutants of E. coli have pleiotropic effects on cell growth; (a) they are defective in the formation of septa between dividing cells after treatment with ultraviolet light and therefore form long filaments, (b) they overproduce the mucopolysaccharide coating of cells, possibly by disrupting the control of operons involved in synthesis of mucopolysaccharide, such as the gal operon, (c) they are defective in the degradation of abnormal proteins. We are investigating the genetic defect in the lon mutants with the aim of determining the interrelationships of these defects.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08714-04 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Mode of Action of a Bacterial Function Involved in Cell Growth Control

Previous Serial Number: ZO1 CB 08714-03 LMB

Principal Investigators: Susan Gottesman, Patsy Trisler, Max Gottesman, Saeko Mizusawa

Other Investigator: None

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	2.6
Professional:	2.6
Other:	0.0

Project Description:

Objectives: Lon mutants are mucoid, UV sensitive, and defective in degradation of abnormal proteins. We are investigating the genetics of these mutations as a basis for future biochemical characterization.

Methods Employed: Standard microbial genetic and biochemical techniques.

Major Findings: (1) Operon Fusions of genes involved in mucopolysaccharide synthesis with lac z and y have been isolated. Insertions of the transposon Tn10 have also been isolated in these genes. Studies on the expression of β-galactosidase in lon^+ and lon^- strains indicates that transcriptional control of genes involved in capsular polysaccharide synthesis is exerted by lon and probably mediated through a gene mapping near 89 minutes on the E. coli chromosome. Transducing phage carrying these fusions have been isolated and can be used as substrates for in vitro studies of transcriptional control. The polysaccharide synthesis genes themselves map near his, and are probably part of the rfb cluster of genes.

(2) A bank of E. coli sequences in a newly developed lambda vector have been constructed and are being screened for the sulA gene. Our genetic experiments indicate that the sulA product is probably the target for lon effects on product UV sensitivity and filamestation. This control has been postulated to act at the level of degradation of the sulA product.

(3) A particular class of rho mutants, defective for phage T4 growth, displays increased degradation of some abnormal proteins. These mutants also interfere with lambda N activity in vivo, although not simply by increasing the degradation of N. A comparison of these rho mutants, their revertants, and other nus mutants (defective for use of N product) is being carried out, with the aim of pinpointing the part of the N-dependent transcription pathway at which these rho mutants are defective.

Significance for Cancer Research and the Program of the Institute: An understanding of the growth control of E. coli may serve as a model for understanding growth control in normal and transformed mammalian cells. Protein degradation is believed to play an important role in the control of mammalian cells; insight into such processes should be gained by further investigation of the process in E. coli.

Proposed Course: Identification of mechanism of control of genes affected by lon, via genetic analysis and in vitro studies of lac fusions; identification of product through which lon effect on polysaccharide synthesis is mediated and analysis of its role in the physiology of the cell; identification of sulA product and its mechanism of regulation by lon.

Publications:

Gottesman, S., Gottesman, M., Shaw, J.E., and Pearson, M.L. Protein degradation in E. coli: The lon mutation and bacteriophage lambda N and cII protein stability. Cell 24: 225-233, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08715-03 LMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Control of Synthesis of a Transformation-Dependent Secreted Glycoprotein

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael M. Gottesman	Chief, Molecular Cell Genetics Section	LMB NCI
Others:	George Vlahakis	Research Biologist	LMB NCI
	Margaret Chapman	Research Biologist	LMB NCI
	Mark Sobel	Senior Investigator	LDBA NIDR
	Stuart Yuspa	Chief, IVPS	LEP NCI

COOPERATING UNITS (if any)

Laboratory of Experimental Pathology, DCCP, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cultured mouse fibroblasts which are transformed by RNA viruses, a DNA virus or a chemical agent, all secrete a 35,000 M_r glycoprotein (major excreted protein, MEP) in large amounts. Nontransformed murine fibroblasts secrete this protein in much lower amounts. These fibroblasts and cultured primary mouse epidermal cells, the target for tumor promoters in vivo, can be stimulated to release MEP by treatment with tumor promoters. We have purified this protein and prepared antisera against it. The protein, of unknown biologic function, undergoes extensive modification prior to secretion. We are studying this system as a model of regulation of protein synthesis, processing and secretion as it is affected by transformation and agents which mimic the transformed state, such as tumor promoters.

PHS-6040
(Rev. 2-81)

Serial No. Z01 CB 08715-03 LMB
1. Laboratory of Molecular Biology
2. Molecular Cell Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Control of Synthesis of a Transformation-Dependent Secreted Glycoprotein

Previous Serial No: Z01 CB 08715-02 LMB

Principal Investigator: Michael M. Gottesman

Other Investigators: George Vlahakis, Margaret Chapman, Mark Sobel, Stuart Yuspa

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981
Total: 1.0
Professional: 1.0
Other: 0.0

Project Description:

Objectives: To determine the mechanism of the control of synthesis, processing, and secretion of the major secreted protein of murine fibroblasts.

Methods: Cell culture; radiolabelling of cell proteins, electrophoretic and chromatographic techniques; immunoprecipitation; in vitro translation of mRNAs; recombinant DNA technology.

Major Findings: (1) We have identified the major excreted protein (MEP) of transformed murine cells. Synthesis and secretion of MEP is increased in all transformed murine fibroblasts and is well-correlated with anchorage independence, and hence with tumorigenicity. Rat cell lines secrete a similar protein which cross-reacts with MEP. Two human cell lines (fibrosarcoma and osteosarcoma) fail to secrete a protein which cross-reacts or biochemically resembles the murine protein.

(2) The purified MEP is extremely heterogeneous, with variation in molecular weight and charge. The molecular weight variation is accounted for by variable glycosylation. The charge variation is due to the presence of multiple mRNA species for MEP, or variations in translation of a single species.

(3) Tumor-promoting phorbol esters stimulate the synthesis and secretion of MEP from non-transformed mouse fibroblasts and epidermal cells. MEP

synthesis is also stimulated in situ in mouse skin treated with the tumor promoter TPA. For fibroblasts, this stimulation, and the transformation-related increase, is due to an increase in levels of translatable mRNA for MEP.

(4) We have found 4 other proteins whose synthesis in mouse epidermal cells is dramatically and specifically increased by treatment with tumor promoters such as TPA. These proteins have been identified on two-dimensional gels and have molecular weights of 25K, 50K, 55K, and 70K.

(5) We have isolated mRNA from transformed mouse fibroblasts which codes for MEP and partially purified it by oligo-dT cellulose chromatography and sucrose gradient centrifugation.

(6) We have developed a radioimmunoassay for MEP.

Significance for Cancer Research: MEP is both a marker of transformation and a sensitive indicator of the presence of at least one tumor promoter. The induction of its synthesis by tumor promoters could serve as a screening test for these agents in the environment. If MEP is found to fill an essential role in tumor growth or metastasis, then specific therapy aimed at neutralizing it could be designed as a model for cancer therapy. Objective 3, Approach 3.5.

Proposed Course: To continue to analyze the molecular mechanism underlying induction of MEP by using cloned MEP cDNAs as probes to measure MEP mRNA levels after transformation and treatment with tumor promoters; to continue to use MEP as a marker of the molecular events involved in tumor promotion and transformation.

Publications:

Gottesman, M.M. and Cabral, F. Purification and characterization of a transformation dependent protein secreted by cultured murine fibroblasts. Biochem. 20: 1659-1665, 1981.

Cabral, F., Gottesman, M.M. and Yuspa, S.H. Induction of Specific Protein Synthesis by phorbol esters in mouse epidermal cell culture. Canc. Res., in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1-CB-08717-03 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
The Biological Role of Glycoprotein Carbohydrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth Olden, Guest Worker	LMB NCI
Other:	Kenneth M. Yamada, Senior Surgeon	LMB NCI
	James B. Parent, Guest Worker	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To investigate the role of endogenous vertebrate lectins in the maturation, metabolism and transport of cellular glycoproteins, we have synthesized phenyl-azido-lactose (PAL), a photolabile derivative of a specific hapten carbohydrate which can act as a photoaffinity inhibitor of lectin activity. This derivative and lactose have similar affinities with respect to endogenous lectin inactivation.

It is internalized by endocytosis, but is not metabolized by the cell. We have also developed a sensitive assay to distinguish between active or PAL-inactivated forms of the lactose-specific lectin, based on the reaction of lectin cysteinyl residues with tritiated N-ethyl maleimide and affinity chromatography.

To study the molecular basis for the increased protease sensitivity of nonglycosylated proteins, we investigated the possibility that asparaginyl residues can participate in the recruitment of proteins for degradation. Reticulocyte lysates catalyze the covalent binding of ubiquitin to polyasparagine, and the ATP-dependent binding of ubiquitin stimulates the proteolysis of this homopolymer by 3-5 fold.

Serial No. ZO1 CB 08717-03 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: The Biological Role of Glycoprotein Carbohydrates

Previous Serial Number: ZO1 CB 08717-02 LMB

Principal Investigator: Kenneth Olden

Other Investigators: Kenneth M. Yamada
James B. Parent

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	3.0
Professional:	2.0
Others:	1.0

Project Description:

Objective: In the present proposal we plan to investigate in depth the precise role of carbohydrates in the intracellular processing, segregation, stabilization and export of glycoproteins.

Specific Aims: (1) The role of carbohydrates in the stabilization of glycoproteins will be examined in vitro by using polyasparagine model peptides and FN.

(2) The role of carbohydrates and/or carbohydrate-binding proteins in the intracellular segregation of glycoproteins will be exmained by studying FN in chick embryo fibroblasts (CEF), acetylcholine receptor (AChR) and acetylcholine esterase (AChE) in muscle cells, and glycoprotein (G) of VSV in mouse L cells. For these studies, we initially plan to use inhibitor of glycosylation (TM) and lectin-specific sugar analogs to disrupt any possible signal between the carbohydrate side chains and intracellular carbohydrate binding proteins. Finally, we will attempt to identify mutants of CHO cells deficient in the lactose binding lectin.

(3) We plan to determine the role of carbohydrate in the expression of the biological and pharmacological properties of the AChR. We are interested in determining if the carbohydrate groups play any role in the function of the receptor as a transducer, in the clustering of receptors at synapses or in neuronal recognition.

Methods: We have inhibited the glycosylation of fibronectin, acetylcholine receptor (AChR), and surface glycoproteins of embryonic muscle cells with tunicamycin (TM) to study the contribution of the carbohydrate moiety to the biosynthesis, processing, turnover and biological activity of these glycoproteins.

The amount of nonglycosylated fibronectin and muscle surface protein exposed on the cell surface was quantitated by iodination of intact cells, SDS polyacrylamide gel electrophoresis, or by a double immunoprecipitation procedure using affinity-purified anti-fibronectin. The amount of AChR exposed on the cell surface was determined by the binding of ^{125}I-alpha bungarotoxin (α-Bgt). The rate of processing and transport to the cell surface was determined by the rate at which newly synthesized glycoprotein becomes trypsin sensitive as it becomes exposed on the cell surface.

To examine the biological activity of fibronectin, we used three approaches. First, we directly measured the effects of fibronectin on cell-cell adhesiveness in a model system that measures hemagglutinating activity. Second, we performed reconstitution experiments in which fibronectin was added to transformed cells deficient in this glycoprotein and measured its capacity to restore the fibroblastic morphology, characteristic of nontransformed cells. Third, we evaluated fibronectin in assays for cell attachment to collagen and cell spreading. For these studies nonglycosylated fibronectin was isolated from cell homogenates of chicken embryo fibroblasts by incubation with anti-fibronectin affinity chromatography.

[^{3}H]-leucine labeled proteolytic fragments of cellular FN will be isolated according to the procedures of Hahn and Yamada (Cell 18: 1043, 1979). We will investigate the protease sensitivity of three major fragments: (1) the heavily glycosylated collagen-binding fragment, (2) the nonglycosylated heparin-binding domain, and (3) the cell-binding domain containing very little carbohydrate. We will also use FN as an authentic protein to investigate the involvement of asparagine residues in regulation of proteolysis.

The biological properties of nonglycosylated AChR will be examined *in situ* in embryonic myotubes by iontophoretic application of acetylcholine and intracellular recording of the depolarization or Na^{+}-flux generated. The redistribution of AChR on the surface of the myotubues during the formation of the neuromuscular junction will be examined by fluorescence microscopy of nerve-muscle cocultures.

Major Findings: The major findings of these studies are that a drastic inhibition of fibronectin glycosylation does not prevent its secretion or export to the cell surface; that the amounts and rates of newly synthesized fibronectin exported to the cell surface are only slightly inhibited by this underglycosylation; that total quantities of fibronectin are substantially reduced in all except intracellular pools, and that this decrease can be accounted for by a specific enhancement in the rate of fibronectin degradation. Similar results were obtained with AChR-an integral membrane glycoprotein, and for the surface glycoproteins of differentiating muscle cells. Thus

carbohydrates may be necessary for stabilizing these proteins against proteolytic degradation, rather than for export or secretion. The levels of FN and AChR can be almost completely restored to normal by incubation with the protease inhibitor leupeptin.

Other major findings obtained are that: (1) the inhibition of the glycosylation of lysosomal enzymes cause them to be processed as secretory proteins (manuscript in preparation), and (2) the binding of ubiquitin to newly exposed, nonglycosylated asparagine residues may be responsible for the enhanced proteolysis of nonglycosylated FN or AChR (manuscript in preparation).

Significance for Cancer Research and the Program of the Institute: Carbohydrates may play a significant role in the stability of cell surface glycoproteins in normal and transformed cells thereby regulating their quantities on the cell surface.

Proposed course: To continue to investigate the role of carbohydrates in the stabilization and segregation of glycoproteins, and also their potential role in membrane recycling.

Publications:

Olden, K., Hunter, V. A., and Yamada, K.M. Biosynthetic processing of the oligosaccharide chains of cellular fibronectin. Biochem. Biophys. Acta 632: 408-416, 1980.

Prives, J. M. and Olden, K. Carbohydrate requirement for expression and stability of acetylcholine receptor on the surface of embryonic muscle cells in culture. Proc. Natl. Acad. Sci. 77: 5263-5267, 1980.

Maheshwari, R. K., Banerjee, D. K., Waechter, C. J., Olden, K., and Friedman, R. M. Interferon treatment inhibits glycosylation of viral protein. Nature 287: 454-456, 1980.

Olden, K., Law, J., Hunter, V. A., Romain, R., and Parent, J. B. Inhibition of fusion of embryonic muscle cells in culture by tunicamycin is prevented by leupeptin. J. Cell Biol. 88: 199-204, 1981.

Parent, J. B. and Olden, K. Role of carbohydrate in cellular recognition and segregation of glycoproteins. J. Supramol. Structure., in press, 1981.

Maheshwari, R. K., Vijay, I. K., Olden, K., and Friedman, R. M. Assay of glycosyltransferase activities in microsomal preparations from cells treated with interferon. In Methods in Enzymology, Volume on Interferon. in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08718-03 LMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic Interactions between E. coli K12 and Bacteriophage λ

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald L. Court, Biologist	LMB NCI
Others:	Ursula Schmeissner, Visiting Fellow	LMB NCI
	Gabriel Guarneros, Visiting Scientist	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have continued our study of λ hin function. It causes several alterations in host physiology (transformation). These may reflect effects of hin on the host membrane. hin has been mapped genetically to two regions on λ. Each of the two genes can independently cause the hin effects.

Studies of mRNA in vivo have revealed that two promoters p_I and p_E are closely related and require cII protein as a positive regulator for initiation. p_I initiates transcripts of the Int gene; p_E the cI repressor gene of λ.

Int transcription from p_I terminates at a rho independent terminator t_I 260 bases beyond int. A second promoter p_L also transcribes int. It is prevented from terminating at t_I by action of another factor, N protein of λ.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08718-03 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Genetic Interactions between E. coli K12 and Bacteriophage λ.

Previous Serial Number: ZO1 CB 08718-02 LMB

Principal Investigator: Donald L. Court

Other Investigators: Ursula Schmeissner, Gabriel Guarneros

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	2.2
Professional:	2.2
Others:	0.0

Project Description:

Objectives: We are interested in a type of transformation of E. coli by λ. To examine this problem we are more precisely mapping the function and isolating specific mutations which inactivate the λ gene(s) hin. Also we are studying the changes wrought on the cellular transport systems and cell membrane itself by λ hin expression. We are cloning the hin gene(s) on a plasmid to study the effect in the absence of other λ genes and in high copy number.

We have cloned the λ tR1 Rho dependent termination site on a plasmid vector and are in the process of isolating new termination mutants in this site. Additionally, we have cloned Rho-independent termination sites and will isolate mutations in these sites also.

Int is transcribed by the promoters p_I and p_L. Int protein is only expressed when made from p_I. Expression from p_L is blocked by a site located distal to the int gene. This site is an RNAse III processing site and inhibition requires cells with intact RNAse III as well as an intact site. We are now trying to determine how processing of an mRNA 250 bases beyond the structured gene inactivates protein synthesis.

This site in addition to being a processing site is a transcription termination site. We are determining whether the same structural signals are recognized by both systems.

Methods Employed: Standard microbial, genetical, biochemical, and recombinant DNA techniques.

Major Findings: (1) hin expression affects amino acid, base, and nucleoside uptake, reduces cAMP levels and increases ppGpp levels in E. coli. It also stabilizes the λ p_L mRNA. Hin does not affect transport mediated by ATP or proton motive force.

(2) The hin gene(s) has been mapped on the DNA--two different genes exist.

(3) cII and cIII proteins are required to allow RNA polymerase to initiate transcription at p_E and p_I in vitro.

(4) The promoters for p_I and p_E have a common cII binding sequence.

(5) p_I but not p_L transcription terminates 260 bases beyond the int gene.

(6) Int expression from p_L is inhibited by a site (sib) 260 bases beyond the gene.

(7) Sib point mutations that eliminate inhibition have been sequenced.

(8) p_L but not p_I RNA in this region is processed by RNAse III in vivo. RNAse III mutants can express int from p_L.

(9) The terminator structure at t_I and the RNAse processing site at sib overlap.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Object 3, Approach 1.

In cancer cells, the expression of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using E. coli and λ as model systems. This understanding might help to prevent conversions of cells to those capable of forming cancers.

Proposed Course: 1. To determine the mechanism of host transformation by phage λ. 2. To determine the mechanism of transcription termination. 3. To determine the mechanism of transcription initiation and control by cII and cIII.

Publications:

Court, D., Brady, C., Rosenberg, M., Wulff, D.L., Behr, M., Mahoney, M., and Izumi, S. Control of transcription termination--A Rho-dependent termination site in phage lambda. J. Mol. Biol. 138: 231-254, 1980.

Schmeissner, U., Court, D., Shimatake, H., and Rosenberg, M. Promoter for the establishment of repressor synthesis in bacteriophage λ. Proc. Natl. Acad. Sci. USA 77: 3191-3195, 1980.

McKenney, K., Shimatake, H., Court, D., Schmeissner, U., Brady, C., and Rosenberg, M. A system to study promoter and terminator signals recognized by Escheria coli RNA polymerase. In Gene Amplification and Analysis, Vol. II, Analysis of Nucleic Acids by Enzymatic Methods, in press, 1981.

Court, D., Gottesman, M., and Gallo, M. Bacteriophage lambda Hin function I. Pleiotropic alteration in host physiology. J. Mol. Biol. 138: 715-729, 1980.

Court, D., de Crombrugghe, B., Adhya, S., and Gottesman, M. Bacteriophage lambda Hin function II enhanced stability of lambda messenger RNA. J. Mol. Biol. 138: 731-743, 1980.

Wulff, D.L., Beher, M., Izumi, S., Beck, J., Mahoney, M., Shimatake, H., Brady, C., Court, D., and Rosenberg, M. Structure and function of the cY control region of bacteriophage lambda. J. Mol. Biol. 138: 209-230, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08719-02 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Development of Recombinant Vectors for Transfer of Genes into Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bruce Howard	Senior Investigator	LMB NCI
Others:	Cory Gorman	Postdoctoral Fellow	LMB NCI
	Raji Padmanabhan	Chemist	LMB NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Gene Regulation Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have used recombinant DNA techniques to construct a series of eucaryotic vectors. These vectors function both in E. coli and in mammalian cells and, thus, may be used to isolate defined eucaryotic genetic segments in E. coli for subsequent transfer to and functional analysis in an appropriate mammalian genetic background. The prokaryotic elements of the constructs derive from E. coli chromosomal DNA, bacteriophage lambda and the plasmid pBR322; the eucaryotic elements derive from simian virus 40. We are currently using this approach to study mammalian promoter regions and RNA processing segments in in vitro tissue culture systems.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08719-02 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland 20205

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Development of Recombinant Vectors for Transfer of Genes into Mammalian Cells

Previous Serial No.: ZO1 CB 08719-01 LMB

Principal Investigator: Bruce H. Howard

Other Investigators: Cory Gorman, Postdoctoral Fellow; Raji Padmanabhan, Chemist.

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	3.0
Professional:	2.0
Other:	1.0

Objectives: Our primary objective in this work is to construct recombinant vectors suitable for cloning in E. coli and virtually any mammalian cell line. With such vectors, mammalian genes and gene segments may be: 1) isolated in E. coli to study at the primary structural level; 2) transferred to mammalian or avian tissue culture cells to study function in a suitable genetic background; and 3) modified to probe for critical regulatory regions.

Methods: Vectors used in recombinant DNA research must provide at least three functional elements: a selectable marker, a mode for stable propagation in the host (integration into the host chromosome or independent replication), and site(s) to accept insertion of foreign DNA sequences. In construction of vectors which carry sets of functional elements appropriate for both E. coli and mammalian cells, we have recombined DNA from several well-characterized sources: the E. coli vector pBR322, bacteriophage lambda, E. coli chromosomal DNA, and simian virus 40. These vectors carry dominant antibiotic resistance markers applicable both in E. coli (ampicillian resistance and chloramphenicol resistance) and in a wide variety of mammalian cells (mycophenolic acid resistance and methotrexate resistance). Propagation in E. coli is dependent upon the ColE1 replication origin of pBR322; after transfer of recombinant DNAs to mammalian cells by the calcium phosphate precipitation method, propagation is dependent upon integration into the host chromosome.

Major Findings: 1.) The parent recombinant vector in this series is designated pSV2. pSV2 combines the origin of replication and ampicillin-resistance marker from the E. coli plasmid pBR322 with a modified simian virus 40 early transcription region. The SV40 early region retains a promoter, intervening sequence and poly(A) addition site, but has been altered by deletion of 75% of T antigen coding sequence (including the AUG initiation codon) and by placement of unique HindIII and BgIII restriction endonuclease sites near the 5' end of the transcribed region. Cleavage by HindIII and BgIII generates cohesive ends for insertion of foreign coding sequences under control of the SV40 early promoter.

A series of coding sequences has been inserted into pSV2, including:

a) The dihydrofolate reductase (dhfr) coding sequence from the E. coli plasmid R388. This dhfr is highly resistant to the folate antagonist methotrexate; accordingly, its transfer into and expression in mammalian cells as part of the recombinant plasmid pSV2-dhfr should confer methotrexate resistance to recipient cells. We have transfected mouse NIH3T3 cells with pSV2-dhfr recombinants, selected and subcloned continuously growing cell lines in methotrexate medium. Demonstration of recombinant DNA in the host chromosomal DNA and of methotrexate-resistant dhfr activity in cell extracts is in progress. Growth of these cells in selective medium is slow and further modifications of the vector and/or selective conditions may be necessary.

b) The xanthine-guanine phosphoribosyltransferase (xgprt) coding sequence from E. coli (R. Mulligan and P. Berg, Science 209: 1422, 1980). Transformation with the recombinant pSV2.xgprt confers upon mammalian cells the ability to utilize xanthine as a substrate for the purine salvage pathway; thus, in selective medium containing xanthine, mycophenolic acid (an inhibitor of inosine dehydrogenase), methotrexate, hypoxanthine and thymidine this recombinant serves as a dominant selectable marker (R. Mulligan and P. Berg, unpublished results). We have shown that this marker may be utilized for stable transformation of Chinese hamster ovary cells, which are widely used in somatic cell genetics. Elements from the pSV2.xgprt recombinant have also been used for construction of a number of other recombinant vectors (see below).

c) The chloramphenicol acetyltransferase (CAT) coding sequence from E. coli. Highly specific and sensitive assays for this enzyme activity have been developed, and CAT does not occur in mammalian cells; therefore, when pSV2-cat recombinants are introduced into mammalian cells (for example, CV-1 monkey kidney cells in in vitro tissue culture), function of recombinant can be rapidly and easily detected. This system should prove valuable for study of mammalian gene segments containing putative promoter and RNA processing sequences. We have removed the SV40 promoter from pSV2-cat and substituted the chick α_2 collagen promoter (H. Ohkubo, C. Gorman, B. Howard, and B. de Crombrugghe, unpublished) and the Herpes thymidine kinase promoter (C. Gorman, M. Dobson, G. Khoury, and B. Howard, unpublished). Study of function and regulation of these promoter sequences is in progress. The

pSV2-cat recombinant also has the potential to serve as a dominant selectable marker for stable transformation of mammalian cells: tissue culture cells may be sensitized to the inhibitory effect of chloramphenicol by growth in fructose or mannose. Since CAT inactivates chloramphenicol, cells in which this activity is coded by integrated copies of pSV2-cat should be resistant to the antibiotic.

d) The galactokinase coding sequence from E. coli (D. Schumperli, B. Howard, and M. Rosenberg, unpublished). Sensitive and rapid assays are available for this enzyme, and although galactokinase activity is present in mammalian cells, the E. coli enzyme may be separated from the endogenous mammalian activity in most instances. pSV2-gal derivatives should prove useful in study of promoter and RNA processing regions as described for pSV2-cat.

2.) A major goal for pSV2-based vectors is cloning of non-selected mammalian genes in E. coli prior to transfer to an appropriate mammalian cell genetic background. Propagation of pSV2 recombinants in E. coli is based on the element pBR322. Unfortunately, a number of large mammalian genomic segments have proved to be unstable when replication is dependent upon the ColE1 origin of pBR322. To circumvent this problem, we have developed derivatives of pSV2-xgprt recombinants in which propagation in E. coli is accomplished by lambda bacteriophage-mediated integration into the bacterial chromosome. Such lambda-SV2-xgprt vectors may be excised and amplified for structural analysis and/or transfer to mammalian cells.

3.) Stable transformation of mammalian cells by pSV2.xgprt and related vectors depends upon integration of the recombinants (by illegitimate cross-over events) into the host chromosome. Since the efficiency of this integration mechanism is low and since recombinants cannot be readily rescued, there has been considerable interest in the possibility of establishing a stable plasmid state in mammalian cells. P. Howley and co-workers have demonstrated that bovine papilloma virus (BPV) is carried as a multicopy plasmid in some mouse cell lines. Preliminary results from a collaboration with that group indicate that BPV/pSV2-xgprt hybrids may also exist in a free, multicopy form. Combination of the BPV replication moiety with the pSV2-xgprt dominant selectable marker should greatly enhance the range of mammalian cell types in which BPV may be used and thereby facilitate the study of expression of mammalian genes and gene segments carried in a stable plasmid state.

Significance for Cancer Research and the Program of the Institute: The availability of the above-described recombinant vectors should find application in:

a) Somatic cell genetic studies involving selection for hybrid cell lines.

b) Study of cloned mammalian promoters. The SV40 early promoter may be excised from pSV2-cat or pSV2-xgprt, and other promoters inserted. Of particular interest to this laboratory will be study of promoters, such as the chicken α_2 collagen promoter, which exhibit altered function in malignantly transformed cells.

c) Transfer of non-selected genes into mammalian cells. Mammalian DNA segments up to about 10 kilobase pairs may be transferred with currently available pSV2 derivatives. Many mammalian genes are small enough to be within the capacity of this vector system; other mammalian gene loci, however, are considerably larger, ranging in size up to at least 50 kilobase pairs. We will investigate the possibility that lambda-SV2 vectors may be used to transfer such large fragments to mammalian cells. A major long-term goal is to construct large DNA fragment genomic libraries. If successful, such an approach would be exceedingly valuable for the isolation of mammalian genes involved in growth control and in the abnormalities of growth related to the malignant state.

Proposed Course: Further work on recombinant vectors will include: 1) identification of additional coding sequences which will serve as selectable markers or readily assayable enzymatic activities; 2) modification of vectors to optimize expression and/or regulation of non-selected genes and cloned mammalian promoters; 3) development of large DNA fragment cloning methods such as the lambda-SV2 system.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08750-01 LMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of Regulatory Proteins in E. coli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sankar L. Adhya Head, Developmental Genetics	LMB NCI
Others:	Elio Gulletta, Visiting Fellow	LMB NCI
	Susan Garges, Microbiologist	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Developmental Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying the structure and function of three regulatory genes of E. coli: rho, crp and adenylate cyclase. We have cloned the rho gene first into bacteriophage λ and then subcloned it into a multicopy plasmid. We have characterized the rho gene by restriction nuclease mapping and by functional analysis. We have also cloned a ts mutation in rho and found that the mutation is an IS1 insertion element.

We have shown that mutations in cya or crp genes suppress some of the pleiotropic phenotypes associated with the ts mutants of rho. We are currently studying these relationships.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08750-01 LMB
Laboratory of Molecular Biology
Developmental Genetics Section
Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Titles: Regulation of Regulatory Proteins in E. coli

Previous Serial Number: None

Principal Investigator: Sankar L. Adhya

Other Investigators: Elio Gulletta and Susan Garges

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	2.0
Professional:	2.0
Other:	0.0

Project Description:

Objectives: The E. coli proteins Rho, cAMP receptor protein, and adenylate cyclase have roles in regulating the expression of many genes in E. coli chromosome. Rho factor is involved in transcription termination. Without Rho, transcription runs through certain signals where normally termination occurs. cAMP receptor protein (CRP) acts with cAMP (the formation of which is catalyzed by adenylate cyclase) to stimulate transcription of certain operons. The nature of this stimulation of transcription is not well understood.

Mutations in the genes for Rho, CRP, or adenylate cyclase result in multiple physiological defects in an E. coli cell. There is evidence that defects in the Rho protein can be counterbalanced by defects in the CRP or adenylate cyclase genes. We are suggesting that there is an inter-relationship among these three regulatory protein and plan to determine at what level (transcriptional, translational, etc.) the relationship lies. We are also characterizing the genes that control these three regulatory proteins.

Methods Employed: Standard microbial, genetic and biochemical techniques. Also employs recombinant DNA technology.

Major findings: (1) Our previously characterized rho mutations are temperature sensitive for growth. It is not known what directly causes the defect for growth in these strains. Starting from a strain which was mutant in the rho gene, mutations in the genes for adenylate cyclase or CRP could partially suppress the temperature sensitivity for growth. In addition, exogenously added cAMP had a deleterious effect on a rho mutant. For these reasons, it is thought that cAMP levels may be

higher in the rho mutants than in a wild-type strain and that this might in some way, be contributing to the temperature sensitivity. Wild-type Rho may act at the level of synthesis of adenylate cyclase, directly or indirectly, to repress its synthesis. The cAMP levels in rho mutant are currently being measured to see if they differ from those of wild-type.

(2) The rho gene and its promoter have been transferred from the bacteriophage in which it was cloned to a multi-copy plasmid. This has enabled a finer physical map of the region to be made. Due to the finer mapping, the general location of the rho promoter on the cloned fragment has been defined. Large amounts of the fragment containing the rho promoter and gene have been purified, and experiments to determine, in vitro, the factors necessary for rho transcription have been initiated.

(3) The rho promoter and gene, when inserted in the multi-copy plasmid, appears to titrate CRP from other known chromosomal site of action. A known CRP binding site (from the lactose operon) cloned in a similar plasmid also titrates CRP, indicating that CRP may be involved in the regulation of rho.

(4) New mutations were isolated which allow transcription to continue through a rho dependent termination point. These mutations were mapped in the rho gene using the bacteriophage carrying the cloned rho gene. These mutants also exhibit a phenotype similar to crp or adenylate cyclase mutants. These strains are currently being analyzed to determine if there is a different gene mutation causing this effect or if a unique type of rho allele is responsible.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 1. In cancer cells the expression of some genes are permanently turned on, i.e., expressed constitutively and some genes are permanently turned off, i.e., repressed. Our studies are aimed to understand the molecular basis of how genes are turned on and off and how genetic regulatory elements interact with each other.

Proposed Course: 1. To determine the complete structure of the three regulatory genes, rho, cya, and crp of E. coli. 2. To determine the regulation of the expression of these genes. 3. To understand the interaction between these genes.

Publications:

Adhya, S., Garges, S. and Ward, D.F. Regulatory circuits of bacteriophage λ. Progress in Nucleic Acid Res. ed. W. Cohn, Acad. Press, Vol. 26, in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08751-01 LMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic Regulatory Mechanisms in Escherichia coli and its Bacteriophage

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sankar L. Adhya Head, Developmental Genetics Section	LMB NCI
Others:	Meher Irani, Visiting Fellow	LMB NCI
	Oscar Reyes, Expert Consultant	LMB NCI
	Laslo Orosz, Visiting Associate	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Developmental Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have continued our study of the expression of the gal operon of E. coli either from the p_1 and p_2 promoters of the gal operon or from the p_L or p_R promoters of a neighboring lambda prophage. The expression of the operon from its own promoter is occluded when gal is transcribed from the p_L promoter.

We are also continuing our study on the nature of transcription termination in E. coli, and of the mechanism of action of the bacteriophage lambda antitermination-function, the product of the N-gene. We are studying the effect of various host mutations that influence the termination-antitermination even. We have found that translational components are involved in transcription antitermination reaction.

Serial No. ZO1 CB 08751-01 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Genetic Regulatory Mechanisms in Escherichia coli and its Bacteriophage

Previous Serial Number: None

Principal Investigator: Sankar L. Adhya

Other Investigators: Meher Irani, Oscar Reyes, Laslo Orosz

Cooperating Units: None

Man Years: October 1, 1980 through September 30, 1981

Total:	3.0
Professional:	3.0
Others:	0.0

Project Description:

Objectives: The gal operon of E coli is normally subjected to negative control by the gal repressor. In addition, the expression of the three cistrons of the gal operon is differentially affected by the presence or absence of the positive control factors, cyclic AMP and CRP. The operon can also be transcribed from a prophage promoter under the influence of the lambda gene N-product. Under these circumstances, the expression of the operon is non-coordinate. We are studying the factors that control gal expression. We are also studying DNA-protein interactions at the gal operator site.

The lambda gene N-product suppresses transcription termination. Our intention is to determine the mechanism of action of N-function.

Methods Employed: Standard microbial, genetic and biochemical techniques. Also employs recombinant DNA technology.

Major Findings: (1) The gal operon in E. coli consists of three structural genes, K, T, and E, the operator-promoter region being located at the E end. The operon has two promoters, p1 and p2. The expression of the various gal cistrons are modulated by an interplay of the two gal promoters, an operator, gal repressor, RNA polymerase and cyclic AMP and its receptor protein. We are currently studying the start points of gal mRNA from p1 and p2 by capping the triphosphate ends with labeled 32P-GTP and then sequencing the labeled RNase T1 fragments.

We have previously shown that the gal cistrons are coordinately expressed from p1 but are dis-coordinately expressed from p2. We have found that coordinacy is controlled at various levels: (i) preferential inhibition of E translation by cylic AMP; (ii) strength of the two promoters and (iii) cyclic AMP control (directly or indirectly) of the intraoperonic transcription termination processes.

(2) A novel in vivo binding assay was developed for isolating gal operator mutants. It involves in vitro mutagenesis of a small fragment of the gal operator-promoter region, cloned into a multi-copy plasmid. An o^+ plasmid makes an wild-type cell gal constitutive by titrating out the gal repressor molecules. Plasmid mutants (o^c) unable to bind the gal repressor have been isolated as inducible cells. These mutations have been studied in vivo by their introduction into chromosomal DNA by genetic manipulations, developed in our laboratory. The mutations have also been studied by DNA sequencing and foot-printing technics. The results indicate an interaction between cyclic AMP-CRP complex and gal repressor on gal operator DNA.

(3) We are currently isolating second site gal operator mutations, which suppress the gal repressor binding inability of the o^c mutations. This is achieved by both in vitro and in vivo mutagenesis. Genetic and biochemical characterizations of these mutants are in progress.

(4) We have isolated and characterized mutations in an RNA polymerase gene that show conditional (presence or absence of rifampicin) discrimination of various promoters in E. coli. We would be studying these mutations by isolating RNA polymerase molecules from the mutant cells and characterizing them in vitro.

(5) Our previously discovered "promoter occlusion" phenomenon has been further investigated. A powerful promoter p_L, located upstream, makes induction of the gal promoter impossible. We hypothesized that a transcription complex from p_L covers the gal promoter, preventing initiation at the latter. This is supported by the observation that reducing the intensity of p_L transcription by a variety of physiological means restores gal induction. We believe that reduced p_L transcription decreases the concentration of the transcription complexes at the gal promoter. We have supporting evidence to suggest that "promoter occlusion" is an important event in phage lambda's life cycle.

(6) We have been investigating the mechanism of action of lambda antitermination function, the N gene product. Our approach involves studying host mutants which affect N activity. In collaboration with David Friedman of the University of Michigan and Herbert Weissbach of Roche Institute of Molecular Biology, we have identified that the host mutants (nus), which prevent N antitermination activity, code for ribosomal proteins. This agrees with our previously proposed hypothesis that translational apparatus modulate transcription termination reactions.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 1. In cancer cells, the expression of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using gal and λ as model systems. This understanding might help to prevent the conversation of normal cells to those capable of forming cancers.

Proposed course: 1. To determine the complete structure of the gal operon. 2. To identify the regulatory molecules involved in turning on and off the various genes of the gal operon. 3. To understand the biochemistry of transcription initiation and termination signals. 4. To elucidate the mechanism of action of the N-gene.

Publications:

Greenblatt, J., Li, J., Adhya, S., Friedman, D., Baron, L., Redfield, B., Kung, H., and Weissbach, H. Evidence that the L factor required for DNA dependent in vitro synthesis of β-galactosidase is the E. coli nusA gene protein. Proc. Natl. Acad. Sci. USA 77: 1991-1994, 1980.

Gottesman, M., Adhya, S., and Das, A. Transcription antitermination by lambda N-gene product. J. Mol. Biol. 140: 57-75, 1980.

Friedman, D.I., Schauer, A.T., Baumann, M.R., Baron, L.S., and Adhya, S.L. Evidence that ribosomal protein S10 participates in control of transcription termination. Proc. Natl. Acad. Sci. USA 78: 1115-1118, 1981.

Merrill, C., Gottesman, M.E., and Adhya, S.L. E. coli gal operon proteins made after ppophage lambda induction. J. Bact., in press, 1981.

Court, D., de Crombrugghe, B., Adhya, S., and Gottesman, M. Bacteriophage lambda hin function II. Enhances stability of lambda messenger RNA. J. Mol. Biol. 138: 731-743, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08752-01 LMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanism of the Transport of Thyroid Hormones into Animal Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sheue-yann Cheng	LMB NCI
Others:	Ryuya Horiuchi, Visiting Associate	LMB NCI
	Ira Pastan	LMB NCI
	Mark Willingham	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using video-intensification fluorescence microscopy, the thyroid hormone, 3,3'5-triiodo-L-thyronine (T_3) has been shown to enter cultured fibroblasts by receptor-mediated endocytosis. Studies using GH_3 cells and monodansylcadaverine, a potent inhibitor of receptor-mediated endocytosis, showed that cellular uptake as well as incorporation of [^{125}I]T_3 into nuclei was inhibited. These results demonstrate that receptor-mediated endocytic pathway of T_3 entry into cells is physiologically significant.

PHS-6040
(Rev. 2-81)

Serial No. ZO1 CB 08752-01 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1980 through September 30, 1981

Project Title: Mechanism of the Transport of Thyroid Hormones into Animal Cells.

Principal Investigator: Sheue-yann Cheng

Other Investigators: Ryuya Horiuchi
Ira Pastan
Mark Willingham

Man Years: October 1, 1980 through September 30, 1981

Total:	2.1
Professional	2.1
Others:	0.0

Project Description:

Objective: To elucidate the mechanism of the entry of thyroid hormones into cells at molecular level and to understand the mechanism of thyroid hormone action on cell growth.

Methods Employed: Synthesize rhodamine-labeled 3,3',5-triiod-L-thyronine (T_3) and study its binding and internalization in cultured fibroblasts and GH_3 cells by video-intensification fluorescence microscopy. Covalently conjugate T_3 to peroxidase, IgG, biotin or dinitrophenyl derivatives and study their intracellular localization by electron microscopy. Use radiolabeled T_3 to quantify the binding and internalization of thyroid hormone by cells.

Major Findings: By video-intensification fluorescence microsocpy, saturable and specific binding of T_3 to plasma membrane of cultured fibroblasts was demonstrated. The entry of T_3 into cells was shown to occur by receptor-mediated endocytosis. By fluorescence photobleaching recovery, T_3 plasma membrane receptor was shown to be proteinaceous in nature.

Monodansylcadaverine, a potent inhibitor of receptor-mediated endocytosis, inhibited the cellular uptake as well as the incorporation of T_3 into nuclei in GH_3 cells. GH_3 cells have ten times more T_3 nuclear receptors than those of fibroblasts and are responsive to T_3 in producing growth hormone. Thus, the inhibition in nuclear entry of T_3 by monodansylcadaverine shows that receptor-mediated endocytic uptake of T_3 is physiologically important.

Cheng, S.-Y., Maxfield, F.R., Robbins, J., Willingham, M.C., and Pastan, I.H.: Receptor-mediated uptake of 3,3',5-triiodo-L-thyronine by cultured fibroblasts. Proc. Natl. Acad. Sci. USA 77: 3425, 1980.

Nikodem, V., Cheng, S.-Y., and Rall, J.E.: Affinity labeling of rat liver thyroid hormone nuclear receptors. Proc. Natl. Acad. Sci. USA 77, 7064, 1980.

Maxfield, F.R., Willingham, M.C., Pastan, I., Dragsten, P., and Cheng, S.-Y.: Binding and mobility of the cell surface receptors for 3,3',5-triiodo-L-thyronine. Science 211: 63, 1981.

Erard, F., Cheng, S.-Y., and Robbins, J.: Affinity labeling of human serum thyroxine-binding globulin with N-bromoacetyl-L-thyroxine, identification of the labeled-amino acid residues. Arch. Biochem. Biophy. 206: 15, 1981.

Cheng, S.-Y., Rakhit, G., Erard, F., Robbins, J., and Chignell, C.F.: A spin label study of the thyroid hormone binding sites in human plasma thyroxine transport proteins. J. Biol. Chem. 256: 831, 1981.

Edelhoch, H., Cheng, S.-Y., and Irace, N.: Luminescences from biological and synthetic macromolecules: Spectroscopic properties of rhodamine B-labeled thyroid hormone (1981) Annals of N.Y. Acad. Science, in press.

LABORATORY OF PATHOPHYSIOLOGY
ANNUAL REPORT SUMMARY
October 1, 1980 to September 30, 1981

The research effort of the Laboratory of Pathophysiology is focused on the following subjects:

1. Pathophysiology of mammary gland and tumors.
2. Biochemical changs related to onset and cessation of lymphocyte proliferation.
3. Structure and function of biological membranes.
4. Effects of ionizing radiations on DNA proteins and bone marrow cells.
5. Pathophysiology of cachexia in tumor-bearing host.

1. Pathophysiology of mammary gland and tumors

The largest effort of the laboratory in terms of manpower is concentrated in this area. Ten interrelated projects have been carried on during the past year.

(1a.) Mechanism of hormone-dependent mammary tumor regression: (Y. S. Cho-Chung, J. Bodwin, T. Clair, and F. Huang)

The principal objective of this project is to understand the events conducive to mammary tumor regression when the host is ovariectomized. Past efforts of the group demonstrated (a) the importantance of cyclic AMP in the regression process, (b) the need of preserving the structural integrity of the cyclic AMP receptor for regression to occur and (c) the phosphorylation of a nuclear protein occuring within a few hours after removal of the ovaries. In the past year it was observed that oral administration of [^{3}H] DB cyclic AMP concentrated in the mammary tumor and mammary gland twice as much radioactivity than parenteral injection. As a consequence, a larger stimulation of cyclic AMP-dependent protein kinase was obtained in the tumor with a larger increase of the 56,000 dalton protein, a constant step in tumor regression. In hormone-independent tumors, the 56,000 dalton protein showed a charge alteration which did not affect cyclic AMP-binding affinity but reduced the self-phosphorylation, and decreased the ability to interact with the catalytic subunit of protein kinase. The changes in phosphorylation of cyclic AMP-dependent protein kinase may influence the activity of RNA polymerase II. Indeed, in regressing tumors where cyclic AMP-dependent protein kinase activity is enhanced, the activity of RNA polymerase II is decreased.

The *in vitro* translational activity of poly A-RNA was reduced during tumor regression, as expected, but the pattern of the products was different in poly A RNA from regressing versus that of growing tumors. The extent of these differences is being investigated.

In another set of experiments the effect of DMBA treatment on the cyclic AMP system of the mammary gland was studied. Within 24 hr of DMBA administration, intracellular cyclic AMP, adenylate cyclase and NAD-dependent-ADP-ribosyltransferase activities were markedly increased while the cyclic AMP-stimulated protein kinase had a decreased activity. The cytosol of untreated mammary gland had a 56,000 dalton cyclic AMP binding protein at higher levels than the treated gland. Concentration of a 39,000 dalton protein, a fragment of the 56,000, was elevated in the gland of the DMBA treated animal. The increase in the proteolytic fragments was

found also in mammary tissue treated in vitro with DMBA. When DB cyclic AMP was given to the animals treated with DMBA, the number of mammary tumors was sharply reduced; the onset was delayed and the changes in the cyclic AMP system was blocked. The preventive action of DB cyclic AMP on oncogenesis by DMBA will be further evaluated.

(1b.) Control of adenylate cyclase activity in normal and neoplastic cells
(Drs. W. Anderson, A. Kraft, L. Nagarajan)

Modification of adenylate cyclase activity was tested in 3 experimental systems (a)10 to 20 fold increase of adenylate cyclase activity was observed in crude NRK membrane preparations incubated in the presence of activated cholera toxin, GTP, ATP, NAD^+ when a protein, approx. 13,000 MW was also present. This protein is removable from the crude membrane preparation by low salt buffer containing EDTA and DTT. (b) The responsiveness of adenyl cyclase to hormones is lost when NRK cells are transformed by Kirsten or Maloney viruses. Under these conditions [^{3}H] dehydrolprenolol was used to assess the behavior of - adrenergic receptors. In the virol infected cells the number of receptors was increased but catecholamines did not enhance cyclic AMP production. It appears that this is the consequence of a defect in the coupling of the receptor to the catalytic subunit of the cyclase. (c) Basal, GTP-and fluoride-stimulated adenylate cyclase showed a progressive increase when F9 line of teratocarcinoma cells were induced by retinoic acid to differentiate into parietal endoderm cells. This differentiation reduces also the adenylate cyclase response to calcitonin and enhances dramatically cyclic AMP production after stimulation with parathyroid hormones. Whether the modified responsiveness to hormones is due to alteration of the GTP coupling mechanism of the cyclase or to changes in the hormone receptor has to be established.

(1c.) Growth and regression of human breast carcinomas
(Drs. S. Shafie and L. Franco)

MCF-7 human mammary carcinoma lines produces tumors in nude mice; treatment of the host by estradiol increases growth 5-6 fold. Tumors failed to develop in ovariectomized nudes or in intact mice treated with 4 hydroxyandostendione, an inhibitor of aromatase. After the tumor has reached a size of 0.8 cm^3 growth progresses without need of estradiol stimulation. Estrogen treatment of nudes depresses NK cell activity while ovariectomy increases NK cell activity. The observation suggest an involvement of NK cells in the growth of MCF-7. The sensitivity of 5 human breast carcinoma cell lines to adriamycin was compared with their estrogen receptor status and growth rate. The following conclusions were reached: (a) ER-negative cells are more sensitive to adriamycin than ER-positive cells (b) rapidly growing cells are more sensitive to adriamycin (c) in nudes ovariectomy enhanced adriamycin sensitivity of all tumors several fold.

(1d.) Control of growth and differentiation of normal and neoplastic mammary cells.
(Drs. W. Kidwell, W. Lewko, J. Zweibel, N. Nolan and R. Katari)

Evidence that hormone dependency in mammary carcinomas is related to the cell composition of the tumor was sought and the following observations were made. (a) Primary NMU tumors are ovarian dependent for growth, consist of both epithelium and myosepithelium, an estrogen receptor positive, synthesize and require type IV collagen for growth and produce a sarcoma growth factor-like substance. Hormone independent transplantable NMU tumors possess none of these properties. The

growth factor produced by the NMU tumors in vivo and in vitro is now being isolated and purified. In response to a growth stimulus the cell membrane of mammary epithelium become enriched of unsaturated fatty acids. Prolactin stimulation also increases the uptake by the membranes of unsaturated fatty acids. Consequently the microviscosity of the epithelial membranes is considerably enhanced, and the production of cyclic AMP in the presence of GTP + iso proterenol is stimulated 9 fold. The unsaturated fatty acids that enrich the epithelial membrane appear to be recruited directly from the mammary fat pad.

The project on the biological significance of poly ADP-ribose synthetase has been continued. Using drosophrola melanogaster evidence was found against the hypothesis that the chromatin bound enzyme is essential in the repair of single strand breaks.

(1e.) Collagen composition and its degradation during metastases.
(Drs. L. Liotta, J. Garbisa, W. Lanzer)

A human condrosarcoma was grown in nude athymic mice. From this tumor human prototype II collagen was isolated for the first time and a source of this material has been established. A neutral protease has been extracted from the media of cultured metastatic tumor cells and purified about 1000 times. The enzyme (70-80000 MN) cleaves specifically both chains of type IV collagen (isolated without pepsin) but not other types of collagen or fibronectin. The reaction gradients indicate a single cleavage in the body of type IV collagen molecule. This collagenase is produced also by human breast carcinoma cells in culture as pro-collagenase activated by trypsin. In the assumption that the ability to destroy the basement membrane is an essential component of the metastasizing capacity of cells, a new assay measuring invasiveness has been developed. Human amnion separates two chambers. In one the cells are seeded, in the opposite chamber chemotactic agents are added. The cell migrating through the amnion are collected by a millipore filter and counted. A correlation between amnion invasiveness and metastasizing capacity of cell populations is being sought.

As a preliminary evaluation of the importance that cell adhesion to tissue components has in the initiation of metastases, laminin was extracted by EHS mouse sarcoma, purified, and iodinated. α-thrombin was formed to selectively cleave the 400 KD (β chain) of laminin whereas plasmin cleaves both the 400 KD and the 200 KD (α chain). Neither type IV or V were degraded. The cleavage products of laminin digestion by either thrombin or plasmin retained the ability to mediate attachment of epithelium to type IV collagen. None of the substrates were degraded by urokinase. A neutral metal protease has been identified which cleaves native type V collagen under conditions when pepsinized type IV collagen or other collagens are not significantly degraded. The enzyme is secreted into the media by cultured M50-76 reticulum cell sarcoma and by leiomyosarcoma cells. The enzyme exists in a latent form that requires proteolytic action to be activated, it has a MW= 80000 approx. and produces specific cleavage products of both A and B collagen chains.

(1f.) Angiogenesis and mammary tumor growth
(Drs. P.M. Gullino and M. Ziche)

The first part of the project aimed at corroborating the hypothesis that acquisition of angiogenic capacity by a cell population, usually deprived of it, indicates an increased risk of neoplastic transformation. The evidence in favor consist of the following observations: (a) in human mammary glands despite a similar morphology of hyperplastic epithelium about 30% has angiogenic capacity, (b) in mouse mammary gland hyperplastic epithelium with high frequency of neoplastic transformation is more frequently angiogenic than epithelium at low risk. (c) Large plastic coverslips produce sarcomas of the subcutaneous tissue earlier and with higher

frequency than smaller coverslips. Long before any sarcoma is present or expected to initiate, the angiogenic capacity of cells attached to the large coverslip is about 5 fold more frequent than that of cells attached to nude coverslips. (d) Skin fibroblasts in culture become neoplastic, i.e. are able to produce a tumor upon transplantation after the 11th passage but angiogenic capacity appears in these cells after the 4th or 5th passage. It appears that viral infection of mammary epithelium induces angiogenic capacity but not neoplastic transformation. The experiments are not yet completed.

The second part of the project aims at understanding the mechanism of angiogenesis. Thus far it has been found that (a) PGE_1 is able to induce neovascularization at a dose lower than any prostaglandins tested, (b) block of PGE_1 by indomethacin prevents angiogenesis, (c) copper ions are concentrated in the tissue ready to be invaded by capillaries, and (d) animals in copper deficient diet do not produce vascularization in the corneal test. The angiogenic capacity of ceruloplasmin and fragments of the protein that carries copper in serum is now being evaluated.

The project on tumor hyperthermia (Mrs. F. Grantham and Mr. D. Hill) has demonstrated the extent of anisotropy of blood distribution within the tumor and the need of relying more on thermal diffusion than thermal convection in hyperthermic treatments.

(1g.) Effects of phorbol esters on cell growth and differentiation
(Dr. D. Salomon)

Neutrophils, cells from a mouse teratoma and mammary epithelium have been used in a set of experiments aimed at defining the role of promoters (TPA) on oncogenesis. TPA was found to inhibit neutrophil chemotaxis induced by formylated tripeptides. The TPA effect is abolished by glucorcorticoids. The activity of a membrane-oxygenated phospholipase A_2 is enhanced by TPA, is decreased by glucorticoids. This decrease is due to the presence of an inhibitor protein (40-50,000 dalton) induced by steroid treatment. Characterization of this protein is ongoing.

Teratoma cells are being grown in a serum-free media supplemented by several factors, and are induced to differentiate into parietal endoderm. The interaction between the factors necessary for growth and tumor promoters are evaluated. Phorbol esters with promoting activity inhibit binding of EGF to teratocarcinoma cells by decreasing the number of receptor sites. Specific phorbol ester receptors have been partially characterized from teratocarcinoma cells and EGF has been found able to modulate induction of phorbol receptors. Using primary cultures of rat mammary epithelium grown in serum free media, it has been established that formation of type IV collagen is necessary for cell growth and EGF_2 dexametasone act as growth regulators by modulating synthesis and/or degradation type IV collagen. The role of phorbol esters on these functions and the consequences on oncogenesis are being evaluated.

The effect of promotion by phorbol esters on the mammary epithelium in vivo is being studied by injection of the promoter either within the excretory ducts of the mammary gland or directly in the parenchyma. No results are available yet.

(1h.) Mechanism of prolactin action
(Drs. R. Knazek, J.R. Dave, and A. Rotondi)

Prolactin binding to rodent liver membranes increased by 60% when hepatocytes were incubated with phospholipase A_2 or lysolecitin and decreased by about 60% when C_3H mice were depleted of essential fatty acids by dietary means or when prostaglandin was blocked by treatment with indomethacin. Enogenous prostaglandin could not overcome these events. On the assumption that these findings were related to

changes in PRL-receptor availability, the fluidity of the microsomal membrane was compared during PRL treatment. It was observed that optimal binding occurred when the phospholipid/cholesterol ratio and the membrane fluidity were maximal. An evaluation of the rigidity of microsomal membranes was also made during ontogenesis. In C_3H hepatic membranes viscosity was highest in the fetus, but fluidity increased rapidly at birth and reached adult level at 44 days.

Prolactin receptors appear as the fluidity of the membrane increases and reach the highest levels also at day 44. PGI_2 and bradykinin were found to increase membrane fluidity in vitro over a narrow concentration range. Available evidence suggest that specific prostaglandins modulate prolactin receptors in vivo via changes in the fluidity of the cellular membranes. This mechanism operates probably in tumors also. The viscosity of regressing DMBA mammary tumors was increased during hormone induced regression. A prostaglandin-receptor assay is being developed to further the study of the apparent role of prostaglandins in modifying membrane fluidity and receptor exposure.

Granulosa cells were also utilized to evaluate the correlation between prolactin and prostaglandin production. Data have been obtained to indicate that euprolactinemic and hyperprolactinemic states modify ovarian steroidogenesis and the ovulatory process by either accenting or suppressing the FSH-related stimulation of prostaglandin synthesis.

(1i.) Growth and differentiation of mammary gland

(Drs. B.K. Vonderhaar, D. Liscia, M. Bhattacharjee, C. Pintus)

One group of experiments was designed to assess whether events occurring during the development of the mammary gland can condition the future pathological history of the aging gland. Diets deprived of appropriate saturated and unsaturated fatty acids are used to obtain adult mice and rats bearing atrophic mammary glands. Development of the mammary gland is then induced with normal diets and during this period the adult animal is manipulted with a variety of hormones. The pathological history of the late-developed gland is then followed. At this time data are collected about morphology and "hyperplasia" of the mammary epithelium. No data pertinent to the major objective are yet available.

In a second group of experiments the role of T_3 and prolactin in mammary gland development and milk-protein production is being studied. α-lactalbumin was purified to homogeneity from mice mammary gland. Fractionation procedures demonstrated that mouse α-LA exists in two forms both with equal activity in the lactose synthetase assay. Antibodies against mouse α-LA have been obtained, unreactive against mouse casein and serum proteins. Using these antibodies, synthesis and secretion of α-LA was found to be enhanced by T_3 in cultured mammary gland. The question whether DNA synthesis was needed for the initation of milk protein production was analyzed using the organ culture of mid pregnant gland. Immunoperoxidase localization of casein and ^{3}HTdR incorporation into DNA were utilized as analytical tools and the conclusion was reached that large incorporation of ^{3}HTdR occurred before casein was produced but only 5.6% of cells were blocked in metaphase while 24% of cells was positive for casein. DNA synthesis appeared necessary for initiation of casein production but number of mitoses did not reflect the event

Extending previous observations on prolactin binding sites, phospholipid metilation by S-adenosyl-L-methionine and the induction of criptic binding sites was studied in liver microsomal membranes. Hypothyroid animals showed 95% increase in criptic binding sites despite a reduction of total binding. Moreover, evidence was gathered showing great stability of the lactogenic receptor when it is bound to the oPRL or hGH. High stability of the lactogenic receptor has been observed to a broad range of pH and high salt concentrations. These properties are now being used to purify the PRL receptor.

(1j.) Expression of milk proteins in rat mammary gland and tumors
(Drs. Qasba, Dandekar, Horn, Devinoy)

Three classes of cDNA clones containing gene sequences for 21-22.5 K daltons rat lactoproteins have been identified. The DNA sequence analysis shows that rat α-LA has 17 extra residues beyond the C-terminus of the α-lactalbumin isolated and sequenced to date from other species. This C-terminal sequence is hydrophobic, proline rich and bears some resemblance to β-casein sequences.

Double stranded cDNAs synthesized from poly(A)RNA enriched for rat casein mRNA sequences were cloned in E.coli after insertion of the Pst I site of pBR322. Recombinant plasmid DNAs carrying structured gene sequences for caseins have been correlated with their corresponding rat milk caseins. Two types of clones for 42K and 25K caseins have been identified which differ in their restriction maps. Poly A^+ RNA from mammary glands at various stages of activity have been translated as a wheat germ system and the products were analyzed. Various classes of RNAs have been distinguished according to the stage of functional activity of the gland.

The effect of ovariectomy on gene expression in the mammary gland of pregnant rats was examined in detail. Within 40 hr after ovariectomy a 60 fold increase in α -LA content of the gland was found despite an insignificant change in total protein content. A qualitative change in specific RNA sequences was also observed. α-LA sequences increased about 10 fold, γ casein and Wp-protein sequences increased about 4 fold but α- and β-casein and K-protein RNA sequences showd little The quantitative differences in α-LA, caseins and wei protein sequences are being evaluated after the host was treated with perphenazine or a pituitary tumor producing mammotropins and also during physiological pregnancy.

2. Biochemical changes related to onset and cessation of normal cell growth
(Drs. H. Cooper, R. Fagnani, and J. Sando)

The study of conditions controlling protein synthesis in lymphocytes constitutes the predominant objective of this project. (a) Intitator $tRNA^{met}$ was found to increase linearly with the protein synthetic rate at all times during the lymphocyte growth cycle. The concentration of $tRNA_i^{met}$ was also kept at a fixed ratio to total tRNA concentration throughout the growth period. During growth activation, however, the relative proportions of 12 other tRNAs changed in various ways in the context of an overall rise in absolute tRNA levels. Work is progressing on the assumption that changes in $tRNA_i^{met}$ concentration may have a regulatory role in protein synthesis. (b) In resting lymphocytes the majority of ribosomes fail to dissociate and do not participate in protein synthesis. When stimulated, however, they dissociate and a factor (elF-3, a complex of 9 proteins) is being produced. Present studies aim at clarifying this event. (c) Growing T-lymphocytes treated with interferon increased synthesis of 8 peptides (I-peptides). Two of them are predominantly in the post ribosomal supernatant and one is in part bound to ribosome-endoplasmic reticulum fraction. Whether this location indicates that this peptide influences protein synthesis is now under investigation. (d) T-lymphocytes can survive several passages in cultures if treated with media derived from lymphocyte and macrophage populations stimulated by mitogens. The T cell growth factor is bound at the surface of the lymphocyte and can be recovered after elution at low pH. Mitogen stimulated T cells in the presence of $[^3H]$ leucine produced radioactive proteins, a small faction of which coincides with peak T cell growth factor activity. The interaction between the surface protein stimulating T cells and protein synthesis is now being investigated. (e) Binding of phorbol ester to lymphocytes enhances their response to growth factors and provokes synthesis of T cell growth factor. The sequence of events triggered by phorbol esters binding to lymphocytes is being investigated. (f) Among the proteins synthesized by lymphocytes after growth stimulation a novel aminoacid has been identified,

lypusine. This aminoacid is formed by the covalent binding of spermidine with a lysine molecule in a peptide chain. This modification is restricted to a single protein (M_2 ca. 18000) and occurs only after growth stimulation but is present in almost all cell lines studied. The H-protein is being studied further.

3. From gene to protein: structure, function and control in eucariotic cells.
(Drs. S.L. Berger, G.P. Siegal, D.M. Wallace, and R.S. Puskas)

Production of T-interferon was obtained from cultured human lymphocytes. Staphyococcal enterotoxin A was used as mitogen. Only about 40% of cultures produced sizable quantities of T-interferon. The mitogen and heterologous serum were indispensable for T-interferon induction but productivity of the culture could not be related to rate of DNA synthesis. At this time the understanding of the conditions necessary to induce optimal production of T-interferon is nebulous.

RNA from cultures with titers > 100 units ml^{-1} were pooled and injected into the oocytes of Xenopus laevis. Only mRNA sedimenting at 18S gave rise to interferon. This 18S-mRNA is larger than the mRNAs coding for either leukocyte or fibroblast interferon. The T-interferon was not neutralized by antibodies to leukocyte or fibroblast interferon.

Oocytes injected with unfactionated mRNA produced T-interferon when the lymphocytes were stimulated with mitogens, and leukocyte interferon when stimulated by virus. T-interferon titles were 1-2% of the leukocyte titers. The level of T-interferon mRNA in cultures treated with staphylococcal enterotonin A has been calculated to be approximately 10 parts for million on a molecular basis.

The effects of interferons on neoplastic cells, particularly on the metastatic process, are being tested.

4. Freeze-fracture observation of normal and neoplastic cell membranes
(Drs. P. Pinto da Silva, B. Kachar, M.R. Torrisi, J. Chevalier, and M.L. Nagueia)

The newly developed method of quick-freezing by a slamming device does not faithfully represent intermediate changes associated with the fusion of membranes during secretion. A new "fracture-label" method has been developed which permits the study of distribution and partition of lectin binding sites and possibly other binding sites. Initial application of the method to erythrocyte membrane shows that Band 3 component, the principal transmembrane protein, partitions preferentially with the protoplasmic A face and during fracture is dragged across the half of the membrane.

A survey of the structure of the prostate gland, in particular of the columnar epithelial cells, was carried on for the first time using freeze-fracture techniques. Besides a comprehensive survey of the conditions necessary for optimal preparations, it has been found that assembly of tight junctional strands along the plasma membranes of the columnar epithelium can be obtained at will, within a few minutes, even in the presence of protein synthesis inhibitors (cyclohexamide) or of metabolic uncouplers (dinitophenol).

The process of myelin formation has been analyzed using freeze-fracture techniques. In regional correspondence with particle clusters in the axolemma, hexagonal rosettes of particles have been observed at the periaxioal region of the Schwann cell plasma. This may indicate an area of functional interaction between axoplasm and the cytoplasm of Schwann cells.

The spatial arrangement of membrane glycoproteins in the platelets has been studied with the fracture label technique. It appears that the Gl 1 protein, which is linked to the adhesion of platelets to the subendothelium, is almost completely partitioned with the external leaflet of the membrane. The Gl 3, involved in the aggregation process, seems to be mostly located inside the external leaflet.

5. Effects of ionizing radiations on nuleic acids, proteins and bone marrow.
(Drs. P. Riesz, K. Makino, M.M. Mossoba, I. Rosenthal and Ms. Uphoff)

(a) The UV photolysis of tryptophan and tryptophan containing peptides in aerated aqueous solutions has been studied by e.s.r. and spin-trapping techniques using T-nitrousbutane as spin-trap. Most of the observed free radicals can be explained in terms of hydrated electrons generated by photoionization tryptophan reacting with the carbonyl group followed by deamination of the N-terminus.

(b) The hydrogen-deuterium exchange reactions in γ-irradiated DL alanine in the solid state were investigated by spin-trapping and e.s.r. using selectively deuterated DL-alanine. Four exchange reactions were found to occur: the first occurs between the hydrogens of the C-2 carbon of the radicals and those of the methyl group of the undamaged molecules, the second reaction takes place between the methyl hydrogens of the radicals and the C-2 hydrogens of nearby molecules, while the remaining processes involve the exchange between the hydrogen atoms of the amino groups and C-2 and C-3 atoms of the deamination radicals.

(c) The direct photoexcitation of pyrimindine bases in D_2D solutions yielded free radicals that could be attributed mostly to D-addition to one end of the 5-6 double band.

(d) γ-radiolysis in the polycristalline state and U.V. photolysis in aqueous solution at 220 nm of several dehydropyrimidines and their derivatives are being investigated by spin-trapping and e.s.r.

(e) U.V. irradiation at room temperature of amino acids and peptides has been studied with light of $h > 300$ nm in the presence of dibenzoylperoxide. This compound is extensively used for treatment of facial acne and is a promoter of carcinogenesis. The radical produced at 313 nm in dimethylsulfoxide were characterized by spin-trapping using 2-methyl-2-nitrosopropane. The predominant reactions were the decarboxylation of the amino acids and the carbonyl-terminal residue in peptides. The volins magety consistently yielded H-atom abstraction radicals. The photo-induced reactions of dibenzoylperoxide with pyrimidines and peptides when exposed to light of $h > 300$ nm suggest the possibility of deleterous effects when topical application of benzoylperoxide is followed by exposure to sunlight.

(f) The physical factors influencing the success of bone marrow replacement following toxic levels of ionizing irradiation have been further investigated by Miss Uphoff. The following conclusions have been reached: (1) The half value layer should no longer be considered sufficient indication of the quality of x-ray, (2) 250 KV x-ray machines should no longer be used as standard for calculating relative biological effectiveness of other radiations, (3) data on males and females should not be combined without first demonstrating that non-dependent sex differences do not occur, and (4) the mean tissue dose obtained with one directional lateral Cs-137 source should not be expected to produce the same biological effect as the uniform tissue dose produced by a two-directional (dorsal-ventral) Cs-137 irradiator.

6. Total metabolism of cancer cachexia
(Drs. S. Morrison, A. Kirkemo)

Work is continuing on the behavioral and metabolic origins of the decline in food intake that is largely responsible for the cachetic decay accompanying tumor growth. Studies on the effect of 4 transplantable tumors on response to lowered environmental temperatures have been completed in rats and the results are being analyzed. A study of the feeding response to insulin in tumor bearing rats has has completed and the results are being analyzed.

Another study on the change in feeding efficiency during tumor growth has been completed and shows that this change is mediated extra-hypothalamically.

Projects on the effect of TPN on survival time of tumor bearing rats and on comparison between enteral and parenteral feeding during tumor growth are underway.

A contract-project at the University of Maryland is concerned with the effects of quantitatively imposed exercise and/or variations in dietary carbohydrate source on tumor growth in rats. The study is not completed. Preliminary data suggest that impaired activity may slow tumor growth.

7. Search of antitumor agents by preparation of amino acid analogs.
(Dr. T. Otani)

Earlier Dr. Otani found that N-benzoyl derivatives of phenylalanine were potent inhibitors of growth of lactobocollus casei 7469. In the past year 5 halogenated benzoyl groups and 2 p-halo-phenylalanines were studied. About 250 of the possible combinations of the various substituted benzoyl groups with the various substituted phenylalanines have been obtained in sufficient purity for microbial studies and 72 have been tested for growth inhibitory activity. The most powerful inhibitor was p-chlorobenzoyl-p-chloro-DL-phenylalanine which has ID-50 of 0.5 mM. The effort of enhancing growing inhibitory capacity by manipulating the chemical structure of amino acid analogs will continue.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00306-18 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Modification of Amino Acids in Search of Possible Anti-tumor Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Theodore T. Otani Research Chemist LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

~~Laboratory of Pathophysiology~~

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

~~NCI, NIH, Bethesda, Maryland 20205~~

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To prepare new amino acids and amino acid analogs and their derivatives by organic chemical methods in search of compounds that destroy cancer cells.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To prepare chemical compounds which preferentially destroy cancer cells by mechanism other than those of agents presently being used in cancer chemotherapy.

The project, initially concerned with developing simple methods of the chemical preparation of β-hydroxyamino acids and with the study of the biochemical properties of these compounds, has been directed in recent years to the search of chemotherapeutic agents active against cancer. The interest has been centered primarily on amino acids, amino acid analogs, and their derivatives thus far, but will be extended to other classes of compounds.

The immediate objectives of the project have been: (a) to prepare various N-acyl and N-aroyl derivatives of amino acids and amino acid analogs in various combinations of the N-acyl group and the amino acid moiety, and (b) to study the relationship between inhibitory activity and the structure of these compounds, as the combinations of the acyl groups and the amino acid analog moieties are varied, and (c) to test these compounds for anti-tumor activity, first in a microbial anti-tumor pre-screen system and, if found to be active, in a mammalian tumor system.

Methods Employed: In most cases, acylation, including aroylation, was accomplished by the Schotten-Baumann procedure, which involves addition of the acylating agent in a 2-phase system sufficiently basic to expose the amino group. In some cases, this was modified by dissolving the appropriate acylating agent in an organic solvent. The contaminating bi-product, the substituted benzoic acid, was generally removed from the reaction mixture by fractional crystallization from carbon tetrachloride, and the product was further purified by crystalization from ethanol, or by precipitation from ethanol-water. In some cases, crystalization was accomplished by repeated cycles of treatment with ethanol, acetone, and petroleum ether.

Microbiological assay was carried out in a riboflavin-supplemented riboflavin assay medium, and the extent of inhibition was determined turbidimetrically. The test organism was Lactobacillus casei 7469.

Major Findings: 1. Earlier we have found that among the many acyl amino acids and amino amino acid analogs we have studied, the N-benzoyl derivatives, especially those of phenylalanine and phenylalanine analogs, were the most potent inhibitors of growth of the selected microorganism. In view of these results, other benzyol derivatives of these amino acids and amino acid analogs having substituents in various positions on the benzoyl ring as well as on the the phenyl ring of the phenylalanine were prepared.

2. In addition to the 12 substituted benzoyl moieties mentioned in the previous annual report, 5 halogenated benzoyl groups, i.e. o-bromobenzoyl, m-bromobenzoyl, p-bromobenzoyl, o-iodobenzoyl, and p-iodobenzoyl groups were studied. In addition to the 14 phenylalanine and phenylalanine analogs mentioned in that report, 2 p-halo-phenylalanines, i.e., p-bromophenylalanine and p-iodophenylalanine, were employed. Hence, the combinations of the various substituted benzoyl groups with the various substituted phenylalanines bring the total number

of possible compounds to some 272. Most of these have thus far been prepared (about 260). Of those that have been prepared, about 250 have been obtained in sufficient purity for microbial studies. The purity has been established by (a) elemental analysis, (b) melting point determination, (c) optical rotation measurement, where applicable, and (d) manometric determination of primary amino nitrogen.

3. Thus far 72 of these compounds have been tested for microbial growth-inhibitory activity.

4. These compounds were studied for their microbial growth-inhibitory capacity as the benzoyl ring-substituted derivatives of 6 amino acid analogs. There were 12 benzoyl ring-substituted derivatives for each of the 6 amino acid analogs. The substituted benzoyl groups were: (1) o-fluorobenzoyl, (2) m-fluorobenzoyl, (3) p-fluorobenzoyl, (4) o-chlorobenzoyl, (5) m-chlorobenzoyl, (6) p-chlorobenzoyl, (7) o-methoxybenzoyl, (8) m-methoxybenzoyl, (9) p-methoxybenzoyl, (10) o-nitrobenzoyl, (11) m-nitrobenzoyl, and (12) p-nitrobenzoyl groups.

The amino acid analogs to which these groups were attached were: (1) o-fluoro-DL-phenylalanine, (2) m-fluoro-DL-phenyalanine, (3) p-fluoro-DL-phenylalanine, (4) p-chloro-DL-phenylalanine, (5) p-nitro-DL-phenylalanine, and (6) β-2-thienyl-DL-alanine.

5. Most of the substituted benzoyl derivatives exhibited inhibition greater than 50% at 1 mg/ml, as follows:

Substituted benzoyl derivatives of:	No. showing more than 50% Inhib.
o-Fluoro-DL-phenylalanine	9 of 12
m-Fluoro-DL-phenylalanine	9 of 12
p-Fluoro-DL-phenylalanine	11 of 12
p-Chloro-DL-phenylalanine	12 of 12
p-Nitro-DL-phenylalanine	11 of 12
β-2-thienyl-DL-alanine	4 of 12

6. In each series, the activity of the o-, m- and p-isomers of an acyl amino acid analog was compared. It was found that depending on the nature of the substituents, there was a different pattern of inhibition among the isomers. For example, when the amino acid analog was o-fluorophenylalanine, and the substituted benzoyl group was fluorobenzoyl group, the o-fluorobenzoyl group was the most active, followed by the m-fluorobenzoyl, and finally by the p-fluorobenzoyl group. But, when the amino acid analog moiety was p-fluorophenylalanine, there was little difference in the activity among the 3 isomers.

7. For the methoxybenzoyl derivatives of o-fluorophenylalanine, the position of the methoxy group on the benzoyl ring was of no importance, as all three isomers showed equal degree of inhibition.

8. Such comparison was made for each of the 6 series of substituted amino acid analog, and it was found that the most powerful substituted benzoyl groups were: the m-chlorobenzoyl, the p-chlorobenzoyl, the m-nitrobenzoyl, p-nitro-

benzoyl, and the o-fluorobenzoyl groups.

9. The least effective substituted benzoyl group was o-nitrobenzoyl group, which seemed to have a nullifying effect on the inhibition, in that the inhibitory activity of the o-nitrobenzoyl derivative of given amino acid analog was less than that of the unsubstituted benzoyl derivative of that amino acid.

10. The most powerful amino acid analogs to which these groups could be attached were: p-chloro-DL-phenylalanine and p-nitro-DL-phenylalanine.

11. The least active amino acid moiety was β-2-thienyl-DL-alanine.

12. Compared on an equimolar basis, most of these substituted benzoyl derivatives of the amino acid analogs thus far studied are much better inhibitors than N-chloroacetyl-β hydroxy-D-norleucine B, the compound taken as a comparative standard in these experiments. Of the 12 derivatives of each series, the following observation was made:

	No. showing 50% Inhibition	No. showing nearly complete inhib.
o-Fluorophenylalanine series	11 of 12	5
m-Fluorophenylalanine series	11 of 12	7
p-Fluorophenylalanine series	11 of 12	4
p-Chlorophenylalanine series	12 of 12	11
p-Nitrophenylalanine series	11 of 12	9
B-2-Thienylalanine series	9 of 12	3

14. A detailed study of the inhibitory capacity of 38 of the most active compounds in which a comparison of the ID-50 value was made indicated that the most potent substituted benzoyl group when combined with the most potent phenylalanine analog yielded the most powerful inhibitor. Hence, the p-chlorobenzoyl moiety when combined with the p-chlorophenylalanine yielded a compound with the lowest ID-50, namely, p-chlorobenzoyl-p-chloro-DL-phenylalanine, which has an ID-50 of 0.50 mM.

Significance to Biomedical Research and the Program of the Institute: That amino acid metabolism may be a susceptible area of action in cancer therapy was demonstrated by the efficacy of asparaginase in certain tumors which required asparagine. Amino acid deprivation, either by the removal of the amino acid or by the substitution with an analog may be effective in inhibiting tumor growth.

The enhancement of activity of amino acid, and amino acid analogs upon acylation appears to be of importance in the cancer program in view of the fact that one serious drawback in cancer chemotherapy has been that of the inaccessibility of the cytotoxic agent to the cell. It is possible that alteration of the molecular charge by acylation, and the introduction of a cytotoxic group, in the form of certain acyl radicals, has provided a molecule that is more permeable to the cell and at the same time, once within the cell, is capable of releasing the cytotoxic agents by the action of intracellular acylases. Objective 6, Approach 2.

Proposed Course of Research:

1. The study of the relationship between chemical structure of the inhibiting compounds and their inhibitory capacity will be continued.

2. The substituted benzoyl moiety studied will be extended to include the o-bromobenzoyl, m-bromobenzoyl, p-bromobenzoyl, o-iodobenzoyl, and p-iodobenzoyl groups, in view of the striking inhibition noted with the p-chlorobenzoyl and the m-chlorobenzoyl derivatives.

3. The study of the amino acid analog moiety will be extended to include p-bromo-DL-phenylalanine and p-iodo-DL-phenylalanine.

4. Derivatives containing the most active benzoyl groups will be coupled with amino acid analogs known to be effective in cancer therapy, such as with sarcolysin.

5. All of these compounds will be tested for inhibitory activity, first in the microbial anti-tumor prescreen and then, if found to be active, in selected mammalian tumor systems.

6. The mechanism of the inhibition noted in these microbial systems will be studied in isolated enzyme systems.

7. The microbial anti-tumor prescreen will be updated to include the compounds recently found to be active against cancer.

8. A study will be undertaken to look into certain products of molds which produce anticancer quinone compounds.

9. The preparation and anti-tumor studies of antimetabolic amino acid analogs coupled with essential fatty acids wil be continued.

Publications:

1. Otani, T.T. and Briley, M.R.: The effect of chloroacetyl derivatives of p-substituted phenylalanines in microbial antitumor prescreen. J. Pharm. Sci. 70, 464-466 (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00941-25 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic and Other Factors Affecting Marrow Transplantation in Irradiated Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. E. Uphoff Research Biologist LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

~~Laboratory of Pathophysiology~~

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

~~NCI, NIH, Bethesda, Maryland 20205~~

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
~~2 1/2~~	1.0	~~1 1/2~~

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In addition to the qualitative difference in x-rays from two different machines using the same filtration and having the same half value layer (HVL) and the dose dependent directional effects of the exposure, other physical factors affect the biological effects of x-irradiation. Dose rate accounts for the difference between dorsal and two-directional exposure but not the differences between alternate dorsal + ventral and ventral + dorsal exposures compared with dorsal and ventral exposures. Dose dependent directional effect also occur following exposure to the higher energy gamma radiation from Cs-137. Conclusions: a) the HVL should no longer be considered sufficient indication of the quality of x-ray; b) 250 kV x-ray machines should no longer be used as a standard for calculating relative biological effectiveness (RBE) of other radiation; c) data on males and females should not be combined without first demonstrating that significant dose dependent sex differences do not occur; d) the mean tissue dose obtained with a one directional lateral Cs-137 source should not be expected to produce the same biological effect as the uniform tissue dose produced by a two directional (dorsal-ventral) Cs-137 irradiator.

Project Description:

Objectives: To investigate genetic and physical factors influencing the success of bone marrow replacement following the administration of toxic levels of ionizing irradiation and/or chemotherapeutic agents used in cancer treatment. The ultimate goal is to devise methods for preventing or controlling lethal graft-versus-host reactions (GVHR) while utilizing possible immunotherapeutic benefits of less severe reactions. To accomplish this goal it has been necessary to investigate and reevaluate many of the universally accepted concepts and practices used by radiobiologists.

Methods Employed: Twenty-six inbred strains and substrains of mice representing the major histocompatibility genotype (H-2) and especially derived substrains are bred and maintained in a closed colony for use in this investigation. Since the mice used for experimentation are residual animals not required for the maintainance of the breeding colony, they are continually available in relatively small numbers and constitute more males than females. To avoid complications arising from age dependent variation in radiation sensitivity all mice are three to four months of age at time of irradiation. Each experiment consists of multiple small groups of mice. Consequently, the significance of uncontrollable variables is reduced. All mice are irradiated between 8 and 10 a.m. to avoid the diurnal variations that occur. The variation observed between three repeated experimental groups governs the total number of animals receiving any given treatment, i.e., the greater the variation in survival the larger the total number of animals treated. For the most efficient utilization of the mouse supply and animal room space, different types of experiments are run concurrently. Following exposure to lethal total-body irradiation from either the double tube 250 kVp therapeutic x-ray machine or the two directional cesium-137 irradiator each mouse receives an intravenous inoculation of bone marrow cells of different genetic disparities between the H-2 genes and immune response genes as well as sex and parity status. In addition, various treatments are given to marrow donors or to the cell suspension to determine the effect of treatment on the protective capacity of the marrow inoculation and on the GVHR. Irradiated mice are observed until death or for 60 days. Marrow inoculated mice in most experiments are observed for 90 days. All high leukemic AKR mice are observed until death as are all the recipients of AKR marrow. Mice developing leukemia are tested by transplanting the neoplasm into both the strains of the marrow donor and recipients to determine their strain of origin.

Major Findings: Among the physical factors already demonstrated to effect the results in this biological system were the quality of the X-rays emitted by two different X-ray machines, using the same filtration and operated under conditions that result in the same half value layer (HVL) of 0.9 mm. Cu. The HVL is universally used to "describe" the quality of X-rays used. It has been assumed that X-rays from machines producing X-ray of the same HVL would produce the same biological effects. However, this assumption has now been demonstrated to be incorrect. At all doses for which comparative data are available using both machines, the Westinghouse Quadrocondex X-ray machine used between 1948-1976 produced both greater and earlier mortality than the presently used Philips RT250 machine under the same experimental condition using mice from the same closed colony. The qualitative differences in the X-ray produced by these machines is also demonstrable in the marrow transplantation experiments.

The addition of an auxillary line voltage regulator does not change the calibrated output of the machines. However, there is a sufficient alteration in the quality of the radiation to produce a significant dose dependent difference in the results of marrow transplantation experiments. In addition, mice irradiated when the auxillary voltage regulator is disconnected but received no marrow (irradiated controls) died later and in some cases there was a reduced mortality compared with the same dose administered when the regulator was in operation.

Another critical factor is the direction of the exposure. Biological systems unlike physical systems may be affected by the rate of exposure which may reflect the effects of repair or physiological changes such as shifts in electrolyte balance or hormonal factors resulting from stress during exposure. It is unlikely that significant repair occurs over the relatively short exposure time of 10-15 minutes to account for the increased survival following uni-directional exposure compared with simultaneous two-directional exposures. The difference between two-directional and dorsal exposure to the same dose was attributable to a time factor and for one strain was equal to a 25R difference in total dose. The difference between dorsal and ventral exposures to the same dose was attributable to a dose reduction by absorption that was equivalent to an additional 25R lower dose to the hematopoietic tissue of the spine and other bones closest to the dorsal surface. Alternate exposure similar to those used in clinical therapy to decrease the surface exposure while increasing the midline dose were tested. Alternate dorsal + ventral exposures, with no time lapse between, was administered over the same total time (plus 2 seconds) as the dorsal exposure. More mice survived than survived a single dorsal exposure. Conversely when ventral exposure preceded dorsal exposure more animals died than with ventral exposure alone but fewer animals died than with the dorsal + ventral exposures. This phenomenon is dose dependent i.e., it is clearly evident in the lower dose ranges. However, as the exposure approaches the lethal range the dorsal and both alternate curves converge while ventral exposure is still less effective even though the exposure times are identical, i.e., a dose that is lethal to all mice under all other conditions will still not kill all mice receiving a ventral exposure. Another critical requirement to demonstrate this phenomenon is the separation of data for males and females.

Radiobiologists have continued to recommend that survival data for males and females be combined when conducting dose response studies. Sex differences in response to ionizing irradiation are strain dependent, i.e., in some strains males are more sensitive than females while in other strains the converse is true. By combining the data for both sexes one avoids the problem of explaining this phenonemon. One contributing factor may be the fact that males are usually larger than females while the other contributing factors are not immediately apparent but it is obviously not a sex hormone effect. When studying dose dependent phenonema males and females must be tabulated separately. Significant sex difference may exist at doses that kill only 10-20% of the mice when data are combined. The significance of this difference disappears as exposures reach the 30 day LD 75 range. Thus, the slopes of the mortality curves for males and females differ. The recommendation should be that data for males and females may be combined only after it is demonstrated that no sex difference in results exists. Subtle effects such as a 6 minute time factor between two directional and dorsal exposures may loose their significance when data for males and females are combined.

To test the validity of the effect of direction of the exposure observed following x-irradiation similar experiments are being conducted using the Gammacell-40 two directional cesium-137 irradiator that was modified to allow for the use of alternate single sources. Mouse holders are placed on a trivet so that the midline of the mouse is at the midline of the chamber. Preliminary data are available for only one strain and may not be typical of all strains. However, with the gamma irradiation the differences between two directional exposure and dorsal exposure may not be as great as those following x-irradiation, i.e., the time factor may be less evident with the higher energy irradiation although the dose rate of 129R/minute is comparable with that of the x-irradiation (126 R/min.). In contrast the difference between dorsal and ventral exposures appear to be greater than that observed following x-irradiation. The difference between dorsal and alternate dorsal + ventral exposures appears to be even greater than following x-irradiation. There is clearly sufficient data available using the cesium source to indicate that directional effects of exposure observed with x-ray is not an artifact.

The high leukemic AKR strain has been used for donors and recipients for reciprocal experiments involving low leukemic CBA mice. At 10-12 month after treatment with 1050 R two-directional gamma irradiation two lymphomas have developed in the AKR mice. The first developed 5 months after treatment and proved to be of AKR origin by the transplantation test. The second developed 8 months after treatment and was a CBA neoplasm. No neoplasms have developed in the CBA recipients of AKR marrow. The higher energy gamma irradiation from Cs-137 has a greater advantage over x-rays in that less gut damage occurs even at much higher doses so there is a minimum of early mortality occurring before the transplanted marrow has become functionally established. Even so a larger group of mice will be required before the comparative incidence of leukemia can be determined.

Significance to Biomedical Research and the Program of the Institute:
Supposition does not become fact by repetition. Progress in science depends upon the replacement of old concepts by new facts. The reluctance to replace accepted dogma as a result of experimental results that cannot be explained in conventional terms has been one of the biggest deterrents to progress in radiation biology. There's a pat answer of all variations ranging from "dirty mice" to "inaccurate calibration" depend upon what best fits the particular situation. Consequently, there is no assurance that the genetic approach of investigating the "acceptions rather than the rule" will be accepted for publication because of their far reaching implications. For example, it is customary to regard the 250 kV x-ray machine as a standard for the determination of the relative biological effectiveness (RBE) of other radiation. RBE is defined by the ratio of doses of x-ray compared with the test radiation that produces equivalent biological effects. With the demonstration that biological systems detect qualitative differences in x-rays where physical systems do not, the 250 kV x-rays should no longer be the standard for calculating RBE. In addition, the HVL, which has been used to "describe" the x-ray beam for purposes of standardizing the output of x-ray machines, is no longer adequate. More precise description of the radiation equipment and the experimental conditions will be required than has been customary heretofore. Some factors affecting the quality of the absorbed dose that have the potential to effect the results are scattered from thick plastic

animal holders or conditions of maximum backscatter, used to decrease the exposure time when one tube is used. Since data describing new findings or new concepts are not accepted unless they can be readily repeated, these experiments have potential importance for all projects using ionizing irradiation.

The direction of the exposure is a critical factor for both x-irradiation and gamma irradiation from Cs-137. The availability of dual opposing x-ray equipment is probably rare. This is one possible explanation for why some of our narrow transplantation experiment have not been readily reproduced elsewhere; i.e., the two directional Westinghouse unit produced a higher and earlier mortality thus mice for marrow transplantation were exposed to a lower total body dose than that customarily used in other laboratories. In the case of cesium irradiators the direction of the exposure becomes of paramount importance. Cesium irradiators, unlike x-ray machines, should produce the most consistent results between different laboratories. However, there are at least two quite different designs for these irradiators. Although the quality of irradiation is not the variable in this case, these irradiators should not be expected to produce comparable results. The Gammacell-40, two directional unit is produced by AECL and should give the most uniform tissue dose particularly if the mouse holder is placed on a trivet the midline of the mouse corresponds to the midline of the irradiation so chamber. In contrast, the Mark I unit by Shephard has a single source located at the side and back of the irradiation chamber. With this lateral exposure variables would be less readily monitored and controlled and a mean tissue dose will be administered. Care must be taken to insure that complete revolutions of the turntable are made for each exposure. When several animals are irradiated at one time, one side of each animal would always be farther away from the source than the alternate side. The demonstration that the dose to the hematopoietic tissue of the dorsal vs. ventral exposure differs by the dose reduction by absorption of the ventral exposure should also be applicable to lateral exposure. In this case, since the spleen is located on the left side of the animal, a dose administered with the left side closest to the source would be analogous with a dorsal exposure. However, when mice are arranged so that the right side of the mouse is closest to the source the effective dose to the hematiepoietic tissue of the spleen may be reduced much as in the case of the ventral exposure. Other factors affecting the comparability of the biological effects between these two units would be the differential absorption of the radiation by bone and the soft tissue of the spleen. In addition, there may be a considerable dose reduction to areas of the skeleton located closest to the side away from the source similar to a ventral exposure. This could result in a greater sparing effect than that observed with dorsal exposure or ventral exposure depend on whether the left or right sides were closest to the source. In any event mice for any given experiment should always be irradiated in the same direction to avoid variation that may cancel interesting radiobiological effects.

Proposed Course of Research: The comparative effect of two-directional, dorsal, ventral and alternate exposures and the dose dependence of this phenomenon will be completed using the Gammacell-40 cesium source. Bone marrow transplantation in high leukemic AKR mice will be expanded to involve exposure to gamma radiation in access of the 1050 R used in the preliminary experiments and to determine the effects of marrow pretreatment on the incidence and genotype

of the leukemias that develop. Efforts will also be made to reproduce and expand experiments involving maternal influences using gamma irradiation for comparison with data previously obtained using the 250 kV x-ray machines. Whether the basic radiobiological experiments can be successfully phased out in favor of the immunogenetic investigations of the type conducted before the Westinghouse x-ray machine was dismantled in 1976 will depend upon the response obtained when our data are submitted for publication.

Publications:

1. Uphoff, D.E. and Law, L.I. X-irradiation and bone marrow transplantation for treatment of murine leukemia: A reevaluation. In Okunewick, J. and Meredith, R. (Eds.) Graft vs. Leukemia in Man and Animal Models. Boca Raton, Florida, CRC Press. pp. 181-190, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00942-22 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effects of Gamma Irradiation on Nucleic Acids and Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Riesz	Chief, Radiation Biol.,	LPP, NCI
Others:	K. Makino	Visiting Fellow	LPP, NCI
	M. M. Mossoba	Visiting Fellow	LPP, NCI
	I. Rosenthal	Visiting Scientist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of ionizing and ultra-violet radiation on nucleic acids and proteins and their constituents are being studied. The modification of radiation damage in DNA by cancer chemotherapy agents of the intercalating and alkylating types is of interest, since such information may be useful in radiation therapy.

The present report includes electron spin resonance (e.s.r.) studies of spin-trapped free radicals produced by γ-radiolysis of amino acids, peptides, nucleic acid bases, and dihydropyrimidines. The direct UV photolyses of dihydropyrimidines were investigated. The benzoylperoxide induced photolyses of carboxylic acids, amino acids, peptides, and pyrimidines were studied in dimethylsulfoxide as solvent.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The effects of ionizing and ultraviolet radiation on biological macromolecules and their constituents are being investigated. For reproductive death, DNA is the target molecule in viruses and is at least a part of the target molecule in bacteria and mammalian cells. Radiation damage to DNA is produced by the "direct effect" through the formation of radical ions, electrons, excited states and neutral free radicals or by the "indirect effect" where radical species formed in the surrounding medium by radiation react with DNA. For water, these species are hydrated electrons, hydrogen atoms and hydroxyl radicals. In the case of radiation damage to the chromosome, radicals formed in the nucleohistone may cause damage to the DNA.

In the chain of events that leads to loss of biological activity, free radicals play an important role. Chemical compounds have been discovered which significantly modify radiation effects. These include: (a) electron affinity sensitizers which act on hypoxic tumor cells, (b) halogenated pyrimidines which are incorporated into DNA and (c) cancer chemotherapy agents of the intercalating or alkylating type which sensitize tumor and normal cells.

Studies of the mechanism of action of radio-sensitizers and radio-protectors are necessary to design improved combinations of chemotherapy and radiation therapy.

An understanding of the mechanisms by which ionizing radiation brings about the loss of biological activity in macromolecules is likely to help in the development of new methods for altering the efficiency of cell killing with possible benefits to radiation therapy.

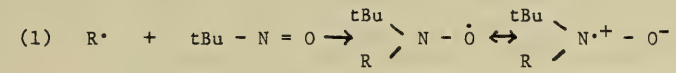
In the last few years it has become apparent that superoxide anion radicals and hydroxyl radicals are found in many biological systems in the absence of either ionizing radiation or UV-photolysis. Recent reports have indicated that hydroxyl radicals are produced in the presence of certain anti-cancer drugs such as bleomycin and adriamycin. The significance of radical reactions is therefore not confined to radiation biology.

Methods Employed: Nucleic acids, proteins and their constituents were γ-irradiated either in the solid state at 2×10^{-5} torr or in aqueous solutions in an 800-curie Cobalt γ-source. Electron spin resonance studies were carried out with a Varian E-9 Spectrometer connected to a Nicolet Lab 80 computer. For photolysis studies at specific wavelengths, a 1000-watt high pressure Mercury-Xenon arc source and monochromator were employed.

Spin-trapping Method:

In the spin-trapping method, the short-lived free radicals react with a diamagnetic scavenger (the spin-trap) to produce longer-lived radicals (the spin-

(the spin-adduct) which can be conveniently investigated by e.s.r. In our studies, t-nitrosobutane (tNB) was employed as the spin-trap, equation (1):



The e.s.r. spectrum of the spin-adduct nitroxide shows a primary triplet splitting due to the ^{14}N nucleus and secondary splittings which usually arise from the magnetic nuclei of the trapped radical R•. From the e.s.r. spectra of the nitroxide, the structure of R. can often be identified. For some experiments in which several radicals were spin-trapped simultaneously, the resulting spin-adduct nitroxides were separated by means of a Waters High Pressure Liquid Chromatograph with a C_{18}-micro BONDAPAK column and water-methanol elution gradients. A capillary flow-detector in the e.s.r. cavity was used to indicate the positions of the free radical peaks.

Major Findings: 1. Spin-trapping and e.s.r. studies of the direct photolysis of aromatic amino acids, dipeptides and tripeptides, and polypeptides in aqueous solutions. Tryptophan and related compounds. (with Y. Lion and M. Kuwabara).

The UV photolysis of tryptophan (trp) and trp-containing peptides in aerated aqueous solutiions has been studied by e.s.r. and spin-trapping techniques using t-nitrosobutane as the spin-trap. The photolysis of trp alone at 290 nm gave rise to the addition of the spin-trap to carbon 3 of the indole ring. A large esr signal from the hydronitroxide spin-adduct was also observed revealing the formation of hydrated electrons. Generally, the photolysis of trp-containing dipeptides generated the deamination radical of the N-terminal amino acid followed by addition to the spin trap. In the case of lysyl-trp, a deamination radical from the side chain of lys was also observed. A sensitization experiment with trp as sensitizer and glycine as substrate lead to the generation of the deamination radical of glycine. Most of the observed free radicals resulting from the photolysis of trp-containing peptides can be explained in terms of hydrated electrons generated by photoionization tryptophan reacting with the carbonyl group followed by deamination of the N-terminus.

2. Hydrogen-deuterium exchange in γ-irradiated polycrystalline DL-alanine (with M. M. Mossoba and I. Rosenthal).

The hydrogen-deuterium exchange reactions in γ-irradiated DL-alanine in the solid state were investigated by spin-trapping and electron spin resonance (e.s.r.) using selectively duterated DL-alanine. Subsequent to γ-radiolysis at 30°C, polycrystalline DL-alanine was dissolved in aqueous solutions of 2-methyl-2-nitrosopropane and the extent of H-D excahnge of the deamination radicals was followed by e.s.r. After formation of the deamination radicals, four exchange reactions were found to occur between the radicals and surrounding undamaged molecules. The first reaction, which occurs between the hydrogens of the C-2 carbon of the radicals and those of the methyl groups of the neighboring molecules, can be followed at room temperature. The three other reactions could be

conveniently monitored in γ-irradiated polycrystalline alanine at 110°C. The first of the other three reactions takes place between the methyl hydrogens of the radicals and the C-2 hydrogens of nearby molecules, while the remaining processes involve exchange between the hydrogen atoms of the amino group and those on the C-2 and C-3 carabon atoms of the deamination radical.

3. Photochemistry of pyramidine bases in aqueous solution as studied by e.s.r. and spin-trapping (with I. Rosenthal and M. M. Mossoba).

The direct photoexcitation of pyrimidine bases in D_2O solutions yields free radicals which could be conveniently identified by spin-trapping with 2-methyl 2-nitrosopropane. Most of the radicals formed were attributed to D-addition to one end of the 5,6 double bond. However, orotic acid and isoorotic acid yielded N(3) centered free radicals, formed by homolytic cleavage of the N-H bond. No indication could be found for a free radical involvement in the photocleavage of cyclobutane-type pyrimidine dimers.

4. Free radicals from dihydrophyrimidines. γ-radiolysis in the polycrystalline state and UV photolysis in aqueous solutions (with M.M. Mossoba and I. Rosenthal).

γ-radiolysis in the polycrystalline state and U.V. photolysis in aqueous solution at 220 nm of several dihydropyrimidines and their derivatives have been investigated by spin-trapping and electron spin resonance. 2-methyl-2-nitrosopropane was used as the spin-trap. The spin adducts of the 6-yl radicals obtained fall into into two categories. Those from dihydro-1-methyluracil, dihydro-6-methyluracil, dihydro-1-ethyluracil and dihydro-1-methylcytosine exhibit a β-nitrogen hyperfine coupling constant (a^{β}_{N}) equal to or less than 2.0 G, while the ones from dihydroorotic acid, dihydrouracil and dihydrothymine showed much larger a^{β}_{N} values (greater than 3.0 G). γ-radiolysis of dihydro-1-ethylthymine generates several radicals with the N(3)-centered radical as teh most stable one. Dihydrouridine gives radicals characteristic of both the dihydropyrimidine ring and the sugar moiety. The same radicals were obtained by γ-radiolysis or U.V. photolysis. For all the 6-yl radicals obtained by U.V. photolysis, a direct photoexcitation mechanism is proposed.

5. Photoinduced Reactions of Dibenzoylperoxide as studied by e.s.r. and spin-trapping (with I. Rosenthal and M.M. Mossoba).

We have studied the room temperature UV irradiation of amino acids and peptides with light of wavelengths significant from an environmental viewpoint, that is, of $\lambda>300$ nm, in the presence of dibenzoylperoxide. The rationale behind this choice lies in the extensive use of dibenzoylperoxide preparations as topical medications for the treatment of facial acne as well as the recently discovered role of dibenzoylperoxide as a promoter for carcinogenesis.

The radicals produced from several amino acids and peptides by UV-irradiation at 313 nm in dimethylsulfoxide solutions in the presence of dibenzoylperoxide were characterized by spin-trapping using 2-methyl-2-nitrosopropane. The most predominent reactions were the decarboxylation of the amino acids and of the carboxyl-terminal residue in peptides. An unusual behavior was exhibited by the valine moiety which consistently yielded H-atom abstraction radicals. No radicals derived from dimethylsulfoxide could be detected under our reaction conditions.

Two mechanistic schemes could explain the experimental results of the present study. Both are initiated by the light absorption by dibenzoylperoxide [reaction (1)]. Subsequently, the nucleophilic attack of the carboxyl group cleaves the excited molecule [reaction (2)]. Alternatively, the excited dibenzoylperoxide homolyses to benzoyloxy radicals [reaction (3)] which, by one electron oxidation of the amino acid carboxyl group, generates the corresponding neutral radical [reaction (4)]. This species dissociates spontaneously to CO_2 and to the trapped decarboxylation radical [reaction (5)].

The question whether one or both reaction pathways are operative, cannot be decided at this time. However, in view of the H atom abstraction capability of benzoyloxy radical, it is tempting to favor reaction (2).

The photoinduced reactions of benzoyl peroxide in dimethylsulfoxide with carboxylic acids, alcohols and pyrimidines were also investigated. For carboxylic acids, the decarboxylation radical was invariably the only radical spin-trapped. For alcohols both hydrogen abstraction radicals and alkoxy radicals were spin-trapped.

In contrast to carboxylic acids, the photoreaction of dibenzoylperoxide with alcohols proceeds by both pathways; these are electron transfers from the hydroxy group to generate the alkoxy radical and H-atom abstraction from the carbon next to the hydroxyl group.

In view of the above suggested mode of acton of excited DBP, that is, the one-electron oxidation, the radicals generated by cytosine and thymine can be easily explained. Thus, one electron oxidation of uracil and its derivatives by $SO_4^{\bullet-}$ was shown to form a cation which most likely loses a proton from the N(1) position to yield the neutral oxidation product with the largest electron spin density at the C(5) position.

The photoinduced reactions of dibenzoylperoxide with pyrimidines and peptides during illumination with light of wavelength (>300 nm) suggest the possibility of deleterious side-effects when topical application of benzoylperoxide is followed by exposure to sunlight.

6. E.s.r. of spin-trapped radicals in γ-irradiated polycrystalline amino acids (with K. Makino).

The radicals produced in polycrystalline L-valine, L-leucine L-isolencine, L-proline and L-hydroxyproline by γ-radiolysis at room temperature were investigated by spin-trapping combined with high pressure liquid chromatography. Deamination radicals and H-abstraction radicals were identified for each amino acid.

Significance to Cancer Research and the Program of the Institute: Studies of the effects of ionizing radiation are of importance in relation to (1) radiation therapy, (2) carcinogenesis, (3) stability of the genetic pool, (4) the suppression of the immune mechanism, and (5) aging. The effects of ionizing radiation on nucleic acids are being studied in order to understand the nature of radiobiological death in normal and tumor cells. The addition of radioprotective and radiosensitizing agents is being investigated so that a therapeutic advantage may be gained. Objective 6, Approach 1.

conveniently monitored in γ-irradiated polycrystalline alanine at 110°C. The first of the other three reactions takes place between the methyl hydrogens of the radicals and the C-2 hydrogens of nearby molecules, while the remaining processes involve exchange between the hydrogen atoms of the amino group and those on the C-2 and C-3 carabon atoms of the deamination radical.

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γ-radiolysis in the polycrystalline state and U.V. photolysis in aqueous solution at 220 nm of several dihydropyrimidines and their derivatives have been investigated by spin-trapping and electron spin resonance. 2-methyl-2-nitrosopropane was used as the spin-trap. The spin adducts of the 6-yl radicals obtained fall into into two categories. Those from dihydro-1-methyluracil, dihydro-6-methyl-uracil, dihydro-1-ethyluracil and dihydro-1-methylcytosine exhibit a β-nitrogen hyperfine coupling constant (a^{β}_{N}) equal to or less than 2.0 G, while the ones from dihydroorotic acid, dihydrouracil and dihydrothymine showed much larger a^{β}_{N} values (greater than 3.0 G). γ-radiolysis of dihydro-1-ethylthymine generates several radicals with the N(3)-centered radical as teh most stable one. Dihydrouridine gives radicals characteristic of both the dihydropyrimidine ring and the sugar moiety. The same radicals were obtained by γ-radiolysis or U.V. photolysis. For all the 6-yl radicals obtained by U.V. photolysis, a direct photoexcitation mechanism is proposed.

5. Photoinduced Reactions of Dibenzoylperoxide as studied by e.s.r. and spin-trapping (with I. Rosenthal and M.M. Mossoba).

We have studied the room temperature UV irradiation of amino acids and peptides with light of wavelengths significant from an environmental viewpoint, that is, of $\lambda>300$ nm, in the presence of dibenzoylperoxide. The rationale behind this choice lies in the extensive use of dibenzoylperoxide preparations as topical medications for the treatment of facial acne as well as the recently discovered role of dibenzoylperoxide as a promoter for carcinogenesis.

The radicals produced from several amino acids and peptides by UV-irradiation at 313 nm in dimethylsulfoxide solutions in the presence of dibenzoylperoxide were characterized by spin-trapping using 2-methyl-2-nitrosopropane. The most predominent reactions were the decarboxylation of the amino acids and of the carboxyl-terminal residue in peptides. An unusual behavior was exhibited by the valine moiety which consistently yielded H-atom abstraction radicals. No radicals derived from dimethylsulfoxide could be detected under our reaction conditions.

Proposed Course of Research: To continue studies on the effects of ionizing radiation on macromolecules of biological importance. The mechanism of radioprotective and radiosensitizing agents and the interaction of radiation and cancer chemotherapy agents will be investigated.

Publications:

1. Bergene, R., Minegishi, A. and Riesz, P. Free radicals in dicarboxylic acids: An e.s.r. study of radical conversions in γ-irradiated single crystals of glutaric acid and glutaric-2,2,4,4-d_4 acid. Int. J. Radiat. Biol. 38, 383-394, 1980.

2. Minegishi, A., Bergene, R. and Riesz, P. E.s.r. of spin-trapped radicals in aqueous solutions of deuterated amino acids and alcohols. Int. J. Radiat. Biol. 38, 395-415, 1980.

3. Minegishi, A., Bergene, R. and Riesz, P. E.s.r. of spin-trapped radicals in γ-irradiated polycrystalline amino acids, N-acetyl amino acids and dipeptides. Int. J. Radiat. Biol. 38, 627-650, 1980.

4. Lion, Y., Kuwabara, M., and Riesz, P. UV Photolysis of aqueous solutions of aliphatic peptides. An e.s.r. and spin-trapping study. J. Phys. Chem. 3378-3384, 1980.

5. Kuwabara, M., Lion, Y., and Riesz, P. E.s.r. of spin-trapped radicals in γ-irradiated polycrystalline nucleic acid constituents and their halogenated derivatives. Int. J. Radiat. Biol. (in press).

6. Kuwabara, M., Lion, Y., and Riesz, P. E.s.r. of spin-trapped radicals in aqueous solutions of 5-halo derivatives of nucleic acid constituents: Reactions of hydrated electrons, hydroxyl radicals and UV photolysis. Int. J. Radiat. Biol. (in press).

7. Kuwabara, M., Lion, Y., and Riesz, P. E.s.r. of spin-trapped radicals from sugars. Reactions of hydroxyl radicals in aqueous solutions and γ-radiolysis in the polycrystalline state. Int. J. Radiat. Biol. (in press).

8. Lion, Y., Kuwabara, M. and Riesz, P. Spin-trapping and e.s.r. studies of the direct photolysis of aromatic amino acids, dipeptides, tripeptides and polypeptides in aqueous solutions. Part 1: Phenylalanine and related compounds. Photochem. Photobiol. (in press).

9. Mossoba, M.M., Rosenthal, I. and Riesz, P. Hydrogen-deuterium exchange in γ-irradiated polycrystalline DL-alanine: A spin-trapping and e.s.r. study. Int. J. Radiat. Biol. (in press).

10. Rosenthal, I., Mossoba, M.M., and Riesz, P. Photochemistry of pyrimidine bases as studied by e.s.r. and spin-trapping. Int. J. Radiat. Biol. (in press).

11. Rosenthal, I., Mossoba, M.M., and Riesz, P. Dibenzoylperoxide-induced photodecaraboxylation of amino acids and peptides. J. Phys. Chem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00944-19 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Total Metabolism of Cancer Cachexia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Seoras D. Morrison	Research Physiologist	LPP, NCI
Others: Aaron Kirkemo	Surgeon	SURG, NCI

COOPERATING UNITS (if any)

Surgical Metabolism Section, Surgery Branch, NCI

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Energy Metabolism Section

INSTITUTE AND LOCATION

NCI, NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is directed towards identifying the causes of the nutritional depletion and general deterioration of the host, known as cancer cachexia, with a view to blocking or reversing these systemic effects of cancer so that the cancer patient could become more accessible to anti-cancer therapies. The approach to this is by investigation of total energy, water and other material exchanges, and of the physiological and behavioral control of food and water intake in normal and cancerous organisms.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: (a) Investigation of the patterns of heat production of rats with concomitant recording of feeding behavior and other activity with a view to finding how the energy expenditure, feeding and general behavior pattern are related to tumor growth, to finding the nature of the loads imposed by a tumor on its host, and to indentifying the causes of cancer cachexia. (b) Investigation of relative changes in water and material exchanges in tissue compartments of the rat during imposed and induced changes in food and water intake and the relation of these to tumor induction and growth. (c) Examination of metabolic effects of total parenteral nutrition and chemotherapy in non-tumor-bearing rats and on the cachexia and decline of food intake induced by tumor growth. (d) Identification of functional sites and causes of breakdown of control of food intake during tumor growth. (e) Development of conceptual models of control of food and water intake and regulation of energy and water exchange and their inter-relationships for normal animals and for the cancer cachectic process.

Methods Employed: The methods of indirect, total, long-term calorimetry, operant conditional responses, placement of electrolytic lesions in or stimulation of the central nervous system, continuous or programmed infusions into unrestrained animals, and methods of orthodox nutritional studies. Computer methods of numerical analysis of serial records of tumor growth, continuous records of gaseous exchange and of change in feeding and drinking patterns in relation to total energy exchange and tumor growth. Sprague-Dawley rats, Buffalo rats and Fischer rats are used in all animal experiments. Most tumor-bearing rats are inoculated with Walker 256 carcinoma, but other transplantable tumors are also used.

Major Findings: Work is continuing on the behavioral and metabolic origins of the decline in food intake that is largely responsible for the cachectic decay accompanying tumor growth. The feeding responses of tumor-bearing animals to stimuli that induce feeding response in normal animals are being examined. Some of the individual components of normal feeding control that are impaired or unimpaired by tumor growth have been identified and quantitatively assessed. Extensive laboratory studies of the effect of 4 different transplantable tumors on response of rats to lowered environmental temperature have now been completed and the very extensive data are being analyzed. A study on the relationship between the behavioral output of feeding and resultant food intake indicates that in normal animals these two variables are partially uncoupled.

An extensive study of the feeding response to insulin in presence of tumor has been completed. Preliminary analysis of the results indicates that interpretation of much earlier work on control of feeding in tumor growth will have to be modified. Another study in the change in feeding efficiency during tumor growth has been completed and shows that this change is mediated extra-hypothelamically.

A project (A. Kirkemo) on the interaction of TPN and effective chemotherapy in a rat tumor model was started but the agent (adriamycin) was found to produce massive hyperlipidemia in rats (an effect not previously reported and not found in man or in mouse tumor models). Projects on the effect of TPN on survival time of tumor-bearing rats and on comparison between enternal and parenteral feeding during tumor growth are under way.

A contract project at University of Maryland (NO1 CB-94327: $36,873.00 for contract year 1980-81) on interaction of imposed physical activity, dietary carbohydrate type and food intake in tumor growth in rats is continuing. Preliminary information usggests that imposed activity may slow tumor growth.

Significance to Biomedical Research and the Program of the Institute: The findings on control of feeding in tumor growth are throwing light on the nature of cancer cachexia and should be utilizable in the development of effective methods for improving the nutritional condition of cancer patients. The section of the National Cancer Plan that the work most closely approximates is: Objective 6 (Develop the means to cure cancers and to retard the progress of cancers not cured). Approach 4 (Enhance the host's ability to eliminate or prevent further development of Cancer). It is also immediately relevant to the 1974 Amendment to the Cancer Act (Collect ....information respecting nutrition programs for cancer patients and the relationship between nutrition and cancer).

Proposed Course of Research: Work in energy and water exchange of tumor bearers will be continued along with study on the interaction of metabolic and behavioral responses of normal and cancerous animals, with particular reference to the reasons for the changes in food and water intake and depletion of host tissues that occur during tumor growth. Work is planned on the interaction of TPN and chemotherapeutic agents in influencing tumor growth and cachexia. Objective 6, Approach 4.

Publications:

1. Morrison, S.D.: Amount of feeding activity and size of meals in free-feeding rats. Physiol. Behav. 25: 893-899, 1980.

2. Morrison, S.D.: Extra-hypothalamic mediation of changes in feeding behavior induced by growth of Walker 256 carcino-sarcoma in rats. Cancer Res. In press.

3. Morrison, S.D.: Control of food intake in experimental tumor growth. Cancer Trt. Repts. In press.

4. Morrison, S.D.: Cold-specific feeding response of rats to cold exposure and the energy density of bodyweight change. J. Appl. Physiol. In press.

5. Morrison, S.D.: Control of food intake in the experimental cancerous host. In Molecular Interrelations of Nutrition and Cancer. In press.

6. Popp, M.B., Morrison, S.D. and Brennan, M.F.: Total parenteral nutrition in a methylcholanthrene-induced rat sarcoma model. Cancer Trt. Repts. In press.

7. Radcliffe, J.D. and Morrison, S.D.: Dietary tryptophan level, food intake and growth in normal and Walker 256 carcinosarcoma-bearing rats. Nutr. Repts. Internat. 22: 563-569, 1980.

8. Radcliffe, J.D. and Morrison, S.D.: Protein quality, food intake and growth in normal and Walker 256 carcinosarcoma-bearing rats. J. Nutr. 110: 2182-2189, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05211-09 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Modification of H1 Histone by Poly(ADPR)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William R. Kidwell	Chief, Cell Cycle Reg. Section	LPP, NCI
Others: Nancy Nolan	Research Biologist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A chromatin bound enzyme, poly(ADP-ribose) synthetase, synthesizes chains of ADP-ribose from substrate, NAD, and transfers these onto a variety of chromosomal proteins. This transfer markedly alters the overall charge of the so-modified proteins and consequently is believed to be involved in alterations of chromatin structure. Poly(ADP-ribose) synthetase on chromatin is activated on chromatin by perturbing chromatin structure such as by chromatin condensing agents or by the introduction of DNA chain breaks. If this appears that poly(ADP-ribose) synthesis is not the inducer of chromatin structural alterations but rather is an intermediate in this process. One proposed role of poly(ADP-ribose) synthetase, DNA strand break repair, is now found to be less likely. Heat shocked drosophila melanogaster cells loose all detectable poly(ADP-ribose) synthetase activity but are still capable of repairing γ-ray induced DNA strand breaks.

PHS-6040
(Rev. 2-81)

Project Description:

Methods Employed: Drosophilia melanogaster cells were propogated in Schneider's Medium and tested for their ability to repair γ-ray induced DNA chain bareaks before and after heat shock at a variety of temperatures. The repair capability as determined by alkaline sucrose gradient sedimentation was correlated with poly(ADP-ribose) synthetase activity remaining after the brief heat shock. Alternatively, the effect of inhibitors of poly(ADP-ribase) synthetase on repair of DNA was tested. The efficacy of the inhibitors in blocking polymer synthesis in vivo was assessed by radioimmunoassay of cell extracts for poly(ADP-ribose) content.

Major Findings:

1. Heat shock effects on poly(ADP-ribose) synthetase in Drosophilia cells. Poly(ADP-ribose) synthetase measurements were made in nuclei from Drosophila cells. ^{3}H-NAD incorporation into the polymer was totally eliminated by incubating at 35°C compared to the activity seen when such nuclei were assayed at the physiological temperature for these cells (25°C). The enzyme was also found to be inactivated in intact cells heat shocked for 5 min at 35°C. This heat shock had negligible effects on cell viability as shown by subsequent cell growth. The effects on cell viability as shown by cell growth. The effects of the heat shock on synthetase were reversible. Activity was not detectable for 1.5 hrs. When recovered exponentially thereafter and ultimately overshot the levels seen in log phase cells.

2. DNA repair in heat shocked cells. During the 1.5 hr period following heat shock when poly(ADP-ribose) activity was missing, the Drosophilia cells were competent in repairing DNA chain breaks induced by γ-irradiation (6.5 Krads.). Based on alkaline sucrose gradient sedimentation analysis, cells with no detectable poly(ADP-ribose) synthetase repaired DNA at a rate equivalent to 60-80% that of non-heat shocked cells.

3. Poly(ADP-ribose) synthetase was activated in vivo by γ-irradiation. Radioimmunoassays of long chain poly(ADP-ribose) content of Drosophilia cells before and after γ-irradiation showed that irradiation increased polymer levels in cells by 3-4 fold. This increase was transient and levels returned to those of control cells by 10 min. after irradiation-long before DNA repair was completed in the cells. The increase in polymer levels in cells in response to irradiation was totally blocked if cells were pretreated with 3-aminobenzamide, a potent inhibitor of poly(ADP-ribose) synthetase. This compound at 2 or 5 mM concentration had no effect on single strand break repair in non-heat shocked, irradiated cells. It is concluded that the purported role of poly(ADP-ribose) synthetase in the repair of single strand breaks in DNA no longer tenable.

Significance to Biomedical Research and the Program of the Institute: Hyperthemia (in heat shocking) is utilized as an adjunct to chemotherapy. Hyperthermia is known to act synergistically, increasing the sensitivity of tumor cells to antitumor agents. Poly(ADP-ribose) synthetase is an exquisitely heat sensitive enzyme, and, as such, is a potential site of action of hyperthermia. If this can be demonstrated to be the case, new combination chemotherapy may be possible using agents such as 3-aminobenzamide, a potent synthetase inhibitor.

Proposed Course of Research: We will extend these studies to other eukaryotic cells to evaluate the possible universality of observations and additionally use the heat shock technique for further probing the possible involvement of poly(ADP-ribose) synthetase in chromatin structural alterations.

Publications:

1. Kidwell, W. R., Stone, P. R. and Johnson, G. S. Poly(ADP-ribose) synthesis and chromatin structure. In Sugimura, T. and Smulson, M. (Eds.): Conference on novel ADP-ribosylations of regulatory enzymes and proteins. Elsevier Press, Inc. New York. pp. 73-84, 1980.

2. Kidwell, W. R., Nolan, N., and Stone, P.R. Variations in poly(ADP-ribose) and poly(ADP-ribose) synthetase in synchrononaly dividing cells. In Hayaishi, O. and Ueda, K. (Eds.), Academic Press, Inc., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05216-10 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotide-Growth Regulatory Mechanism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y.S. Cho-Chung	Chief, Cellular Biochemistry Section	LPP, NCI
Others:	F. Huang	Expert	LPP, NCI
	L. Kapoor	Visiting Scientist	LPP, NCI
	T. Clair	Chemist	LPP, NCI
	B. Berghoffer	Biologist	LPP, NCI
	J. Katz	Chemist	LPP, NCI
	C. Shepheard	Biologist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cellular Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Growth arrest of hormone-dependent mammary carcinomas was achieved by orally administered DBcAMP. Total-and bound-radioactivities were 2-fold higher in the tumors and mammary gland when [^{3}H]DBcAMP was administered orally as compared to s.c. administration. Orally administered DBcAMP elicited the increase of cAMP-dependent protein kinase in the regressing mammary carcinomas. Studies of RNA-polymerase and in vitro translation of polyA RNA isolated from growing and regressing mammary carcinomas suggested the action of cAMP at the nuclear level in the inhibition of mammary carcinoma growth.

PHS-6040
(Rev. 2-81)

Project Description:

Methods Employed:

i. Tumors: Primary, 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary carcinoma and transplantable MTW9, DMBA #1, MT13762, and MTW9A mammary carcinomas were used.

ii. DBcAMP and arginine treatments: DBcAMP 2.0 ~ 20 mg ± 50 ~ 200mg L-arginine .HCl/1.0 ml H_2O/200 g rat, 3 times/week, orally.

iii. Photoaffinity labeling of cAMP-binding protein: The photoactivated incorporation of 8-N_3-[^{32}P] cAMP into cytosol or nuclear proteins was performed by the method of Pomerantz et al.

iv. Eudogenous phosphorylation of cytosol proteins: Performed under conditions favoring self-phosphorylation of protein kinase type II regulatory subunit.

v. Protein kinase assay: The activity was determined by measurement of the incorporation of ^{33}P from γ-labelled ATP into histone ± 10^{-6}M cAMP.

vi. Two-dimensional gel Electrophoresis: The electrophoretic separation procedure was as described by O'Farrell. First dimension isoelectric focusing (IEF) on polyacrylamide disc gels containing 8M urea and then to second dimension electrophoresis on SDS-polyacrylamide slab gels.

vii. RNA-polymerase Assay: followed the method of Schmartz et al and Jungmann et al. Multiple forms of RNA polymerase were separated by preparative isoelectric focusing, DEAE-Sephadex, DE-52, CM-sephadex and phosphocellulose column Chromatography.

viii. In vitro translation: Total poly A containing mRNA was isolated from tumors by the method of Deeley et al. In vitro translation system of both rabbit reticulocyte lysate and wheat germ extract were used. Total translation products were analyzed by SDS-polyacrylamide gel electrophoresis.

Major Findings:

I. Growth arrest and cAMP-dependentent protein kinase

A. Growth arrest by Orally administered DBcAMP

1. Growth of hormone-dependent mammary carcinomas was arrested by orally administered DBcAMP and this growth inhibitory effect was enhanced by L-arginine. Following oral administration of DBcAMP (2mg/200g rat)+ L-arginine (200mg/200$_g$rat), 3 times/week, tumors regressed to 50% of their initial size within 2 weeks and many tumors regressed completely after 3 weeks.

2. Total and bound radioactivities in tumors and mammary glands were appreciably higher than in other rat tissues after oral administration of [^{3}H] DBcAMP.

3. Bound radioactivity in tumors was 2-fold higher when [^{3}H] DBcAMP was administered orally as compared with the s.c. administration.

4. Cyclic AMP-binding and cAMP-dependent protein kinase activity in tumor appreciably increased following oral administration of DBcAMP.

5. A close correlation was found between the increase of the 56,000 dalton cAMP-binding protein, the regulatory subunit of cAMP-dependent protein kinase type II and tumor regression following oral administration of DBcAMP.

B. Charge alteration of cAMP-binding protein in hormone-independent Mammary Carcinomas

1. The molecular species of cAMP-binding protein present in hormone-dependent (HD) (DBcAMP-responsive) and -independent (HI) (DBcAMP-unresponsive) mammary carcinomas were identified and characterized by the use of the photoaffinity ligand, 8-N_3-[^{32}P]cAMP.

2. The 39,000 dalton cAMP binding protein, the proteolytic fragment of the 56,000-dalton cAMP-binding protein, was found in large amounts in most tumors examined, suggesting a high proteolytic activity in tumors in vivo .

3. The 48,000-dalton and 56,000-dalton cAMP-binding proteins, the regulatory subunits of cAMP-dependent protein kinase, types I and II, respectively, were found in varous amounts in both HD and HI tumors.

4. On two dimension gel electrophoresis, however, the 56,000-dalton protein of hormone-independent tumors exhibited a charge alteration which did not affect the cAMP-binding affinity and correlated with the decreased self-phosphorylation of the binding protein.

5. The decreased self-phosphorylation of the 56,000 dalton protein of HI tumors correlated with decreased ability of the cAMP binding protein to interact with the catalytic subunit of protein kinase.

These results suggest that the charge alteration of cAMP binding proteins, which appears to occur at a site remote from that of cAMP-binding may be associated with the hormone-independency (DBcAMP-unresponsiveness) of mammary carcinomas.

II. Phosphorylation and dephosphorylation of RNA polymerase in Mammary Carcinomas

1. Multiple forms of RNA polymerase were present in both growing and regressing MTW9 mammary carcinomas.

2. Total activity and template specificity of polymerase in both growing and regressing tumors were similar.

3. The polymerase II (γ-amenitin sensitive) activity in regressing tumors was inactivated under conditions for cAMP-dependent protein kinase reaction, whereas the enzyme activity in growing tumor was not affected by the kinase reaction.

4. The polymerase II activities of both growing and regressing tumors were activated by treatment with phosphorprotein phosphatase and the activation was reversed by the protein kinase.

The results suggest that the RNA polymerase II in MTW9 mammary carcinomas is regulated by phosphorylation and dephosphorylation reaction and that cAMP-dependent protein kinase may play a specific role in this process.

III. Genetic transcription in growing and regressing mammary carcinomas

1. Growing and regressing (up to 5 days after ovariectomy) DMBA-mammary carcinomas contained similar level of polyA RNA that exhibited in vitro translational activity: as regression proceed, however the tumors contained less polyA RNA and the translational activity declined.

2. The SDS-poly acrylamide gel electrophoretic pattern of the total translational products from regressing tumors was markedly different from that of growing tumors as early as 6 hr post ovariectomy.

3. In the regressing tumors, the in vitro translational product of 50,000~60,000-dalton species disappeared and 30,000~40,000-dalton species reappeared.

Significance to Cancer Research and the Program of the Institute: (NCP Objective #6, Approach #3. These studies contribute to the understanding of the molecular mechanism of cAMP-mediated tumor regression. Orally administered DBcAMP triggered tumor regression by activation of the cAMP system in the tumors. The action of cAMP at the nuclear level is suggested by the data of RNA-polymerase and in vitro translation of polyA RNA. These results together with our previous studies strongly suggest the therapeutic usefulness of DBcAMP + arginine for breast cancer in humans.

Proposed Course of Research: To extend the investigation on the mechanism of cAMP action on growth control, the following proposal is made:

1. Characterize the molecular form of cAMP receptor complex that enters nucleus by the use of site specific anti-cAMP-dependent protein kinase antibodies.

2. Cytoimmunochemical mapping of cAMP-dependent protein kinase-subcellular localization during growth and regression of mammary tumors.

3. Assess the function of cAMP receptor protein in the nuclei in relation to gene translation and transcription.

4. Assess the relationship between nuclear protein phosphorylation, RNA-polymerase activity and gene modulation.

5. Identify the molecular species of in vitro translational products of growing and regressing tumor mRNA

Publications:

1. Cho-Chung, Y.S.: On the mechanism of cyclic AMP-mediated growth arrest of solid tumors. In: Hamet, P. and Sands, H (Eds): Adv. Cyclic Nucleotides Res. New York, Raven Press, 1980, vol. 12, pp. 111-121.

2. Cho-Chung, Y.S.: Cyclic AMP and mammary tumor regression. Cellular and Molecular Biol. 26: 395-403, 1980

3. Cho-Chung, Y.S., Clair, T., Bodwin, J.S. and Hill, D.M.: Arrest of mammary tumor growth in vivo by L-arginine: Stimulation of NAD-dependent activation of adenylate cyclase. Biochem. Biophys. Res. Commun. 95: 1306-1313, 1980.

4. Cho-Chung, Y.S.: Cyclic AMP and its receptor protein in tumor growth regulation in vivo. J. Cyclic Nucleotide Res., 6: 163-177, 1980.

5. Cho-Chung, Y.S.: Mode of cyclic AMP action in growth control. In: Leung, B.S. (Ed.): Hormonal Regulation of Experimental Mammary Tumors. Pergamon Press, in press.

6. Cho-Chung, Y.S., Clair, T., Schwimmmer, M., Steinberg, L., Rego, J.A. and Grantham, F.: Cyclic AMP receptor protein in hormone dependent and independent rat mammary tumors. Cancer Res. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05219-10 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

In vitro Simulation of Hormone-dependent Mammary Tumor Regression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.A. Knazek	Senior Investigator	LPP, NCI
	S.C. Liu	Chemist	LPP, NCI
	J.R. Dave	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)

L. Richardson (IPA) Univ. of Va. School of Medicine

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cell Cycle Regulation

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	1.25	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Alterations of the hormone-receptors on or within cells will modify the response of target tissues to various hormones thus serving to control cellular growth or function. We have shown that the number of detectable prolactin receptors is controlled in vivo by the circulating levels of prolactin or growth hormone by a positive feedback mechanism. Inhibition of in vivo prostaglandin (PG) synthesis by either enzymatic blockade or precursor depletion results in a loss of existing PRL receptors and prevents their induction by PRL. As membrane fluidity increases in a variety of hormonal, dietary or developmental states, the number of PRL receptors also increases. This has been shown in vitro to be achieved by PGI_2 over a very narrow concentration range. It has also been demonstrated that PRL alters PG synthesis in vivo. These data suggest that PRL up-regulates its own receptor by modifying target membrane fluidity and that this may occur through modification of prostaglandin synthesis. These studies have been extended to the DMBA rat mammary tumor, with the regressing tumor membranes more viscous than those of the growing tumor. An assay for PG receptor has been developed.

Project Description:

Objectives: Reproduce and study in vitro, the hormone-dependent regression process of mammary tumors in vivo.

Methods Employed: A radioreceptor assay for prolactin and growth hormone receptors is used to determine the ability of cell membranes to bind these polypeptide hormones.

Assay techniques for prostaglandin E and $F_{2\alpha}$ have been devised consisting of extraction from incubation buffers by organic solvents, silicic acid double column chromatography, and specific radioimmunoassay. High pressure liquid chromatography is used to quantitate prostglandin intermediates synthesized by various tissues.

Cell membrane fluidity was determined by fluorescence polarization. This was accomplished by measuring the ease of rotation of the fluorescent lipid probe diphenylhexatrine (DPH), that had been inserted into the membranes in vitro. The polarization constant was then translated mathematically into viscosity.

Major Findings: PRL receptors can be induced in the rodent liver by in vivo injections of either purified bGH or bPRL. GH was shown to induce but not to bind to the receptor whereas PRL did both. These studies, plus the observation that induction occurred even if protein synthesis had been blocked, indicated that the induction was being achieved by exposing or altering receptor in such a way that its binding site was made available to PRL. Increases in the number of PRL binding sites could also be achieved by direct alteration of the phospholipid bilayer. This was accomplished both in vitro and in vivo. The PRL binding capacity increased by 60% when hepatocytes were incubated with either phospholipase A_2 or lysolecithin but decreased by 60% when C3H mice were depleted of essential fatty acids by dietary means, a loss that could not be reversed by treatment with exogenous PRL. Blockade of prostaglandin synthesis by in vivo treatment with indomethacin also reduced existing hepatic-PRL receptors in a dose-responsive fashion that, again, could not be overcome by exogenous PRL injections.

Modification of the prolactin receptor could therefore be achieved by changes in the character of the lipid bilayer of target membranes, an effect that seemed related to both the presence of prostaglandins and the status of the lipid bilayer in which the receptors are considered to float. Since prolactin induces its own receptor in vivo, studies were undertaken to determine if this could be a result of changes in membrane fluidity with concommitant exposure of preexisting receptors. Doses of 0, 0.5, 5, and 50 µg oPRL were injected s.c. hourly into hypophysectomized rats for the 48 hours prior to sacrifice study of microsomal membranes prepared from the livers of these animals revealed their phospholipids /cholesterol ratio and membrane fluidity to be maximal in the 0.5 µg PRL group and minimal in the 50 µg PRL group. Binding of oPRL was detectable only in the most fluid group, i.e., those treated with 0.5 µg oPRL which provided near-physiologic levels of oPRL. These data indicate that prolactin modifies the viscosity of hepatic membranes in vivo and that this phenomenon might be responsible for the auto-regulation of detectable oPRL receptors.

Measurement of the microviscosity and the hepatic membranes of C_3H mixed revealed an extremely rigid membrane in the fetus that rapidly becomes highly fluid post-natally, reaching a viscosity nadir 21 days after birth. Subsequently, the viscosity of the membranes increases rapidly and reaches adult levels by 44 days of age. PRL receptors, meanwhile, appear for the first time during the period in which the membranes are most fluid and also reach adult levels by 44 days of age. These changes in both receptor levels and fluidity suggest that such age-related modifications of cellular membranes are interrelated and that changes in the membrane fluidity during development may be of prime importance in modulating the ability of cells to respond to endogenous and exogenous stimuli.

Additional studies demonstrated that PGI_2 increased membrane fluidity in vitro over a very narrow concentration range, a maximum effect of 60% at 10^{-7}M with higher concentrations increasing the viscosity back to control levels. This viscosity change was maximal after 30 min incubation and reflected an increase in the number of receptor sites as determined by Scatchard analyses. On the contrary, when mice were depleted of the prostaglandin precursors by placing them on diets devoid of the essential fatty acids, the viscosity of the hepatic membranes increased with a concommitant decrease in the number of prolactin receptors.

Additional studies showed that bradykinin, a stimulator of phospholipase A_2 activity, simultaneously increased PRL binding capacity and fluidity of murine hepatic membranes in vitro. This occured over a very narrow concentration range, the maximal effect being seen at 5 µg/ml. It was subsequently shown that arachidonic acid could reproduce this effect, again, over a narrow concentration range in vitro.

Cholesterol is known to increase micro-viscosity when inserted into biological membranes. Diets containing corn oil (5% w/w) or medium chain triglycerides (5% w/w) comprised the control (CON) and essential fatty acid deficient (DEF) diets, respectively. Cholesterol (0.1% w/w) was added ⊕ or not added ⊖ resulting in 4 diets: CON ⊕, CON ⊖, DEF ⊕, and DEF ⊖. Mammary gland development was evaluated by whole-mount techniques at various times after initiation of the dietary regimens at weaning. While the animals from the first three groups acquired alveolar structures at the same rate, those in the DEF ⊖ group began to regress at ~ 21 weeks of age and were without alveoli by ~ 30 weeks of age. The DEF ⊕ animals, however, never developed any alveolar structures. This supports the observation that the most rigid membrane states are less likely to respond to trophic hormonal stimuli.

It appears, therefore, that modification of hepatic microsomal membranes by either the removal of prostaglandin precursors or their supplementation with PGI_2 induces either an increase or a decrease, respectively, in the membrane microviscosity. The data suggest that specific prostaglandins may modulate the number of prolactin receptors in vivo and that this may occur by modifying the fluidity of the lipid bilayer and the subsequent ease with which the receptors move within that matrix. This may prove to be a mechanism of physiologic significance in the minute-to-minute regulation of membrane associated receptors in vivo. Study of rat mammary tumors demonstrate that this hypothesis can be extended to neoplastic tissues: the viscosity of DMBA tumor membranes 5 days after ovariectomy were 2.2 times more viscous than those of a comparable growing tumor from an intact animal.

Thus, the apparent role of prostaglandins in the modulation of cell membrane fluidity with resultant changes in plasma membrane receptors, suggests that cellular response to prostaglandins might be controlled further by alteration in their ability to respond to various prostaglandins. To this end, a prostaglandin-receptor assay was devised wherein microsomal membranes were obtained from DMBA rat mammary tumors, incubated with ^{3}H-PG (E_2 or $F_{2\alpha}$) $\pm$ unlabelled PG. The unbound PG was separated from the membrane-PG complex by rapid filtration. Binding was rapid, reaching a plateau after 15 minutes, reversible with excess unlabeled PG, and saturable.

Significance to Biomedical Research and the Program of the Institute: Demonstration that prolactin receptors are induced by growth hormone indicates that this hormone may play an important role in the responsiveness of mammary carcinoma to hormonal stimuli. The fact that changes in membrane fluidity are induced by prolactin may help to explain the mechanism by which prolactin induces its own receptor. The requirement for essential fatty acids and prostaglandins to modulate the prolactin receptors indicates that the lipid stores play an important role in maintaining the responsivity of target tissues to circulating hormones. These studies support and explain, in part, the demographic correlation of mammary carcinoma with high dietary fat intake throughout the world.

Proposed Course of Research: The role of prostaglandins in the induction of the prolactin receptor and prolactin action will continue to be studied *in vivo* and *in vitro*. The role of prostaglandins in the development of the mammary tumors and mammary glands will also continue to be studied using radioimmunoassay and HPLC techniques.

Publications:

1. Nissley, S.P., Knazek, R.A. and Wolff, G.L. Somatomedin activity in sera of genetically small mice. Hormone and Metab. Res. 12: 156-164, 1980.

2. Knazek, R.A., Watson, K., Lim, M., Cannazzaro, A., Christy, R.J. and Liu, S.C. Prostaglandin synthesis by murine mammary gland is modified by the stage of estrus cycle. Prostaglandins 19: 891-897, 1980.

3. Knazek, R.A. and Liu, S.C. The effects of dietary essential fatty acids on murine mammary gland development. Cancer Res. In press.

4. Dave, J.R. and Knazek, R.A. Prostaglandin I_2 modifies both prolactin binding capacity and fluidity of mouse liver membranes. PNAS. 77: 6597-6600, 1980.

5. Onedera, T., Tonedo, A., Ray, U.R., Jenson, A.B., Knazek, R.A., Notkins, A.L. Virus-induced diabetes mellitus. II. Polyendocrinopathy and autoimmunity. J. Exptl. Med (in press).

6. Knazek, R.A., Liu, S.C., Dave, J.R., Christy, R.J., and Keller, J.A. Indomethacin causes a simultaneous decrease of both prolactin binding and fluidity of mouse liver membranes. Prostaglandins and Medicine (in press).

7. Dave, J.R., Knazek, R.A., and Liu, S.C.: Prolactin modifies the fluidity of rat liver membranes. Biochem. and Biophys. Res. Comm., Vol. 100, pp. 45-51, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08205-10 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Application of the Capillary Culture Technique to Polypeptide Hormone Production

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. A. Knazek	Senior Investigator	LPP, NCI
Others:	P. M. Gullino	Chief	LPP, NCI
	L. A. Liotta	Expert	LPP, NCI
	R. S. Balaban	Staff Fellow	LKEM,NIH,BLD
	P. M. Howley	Senior Investigator	LP, NCI
	N. Sarver	Visiting Scientist	LP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cell Cycle Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The artificial capillary culture technique provides a pericellular microenvironment that closely resembles the in vivo state. This may allow normal cells to maintain their differentiated functions for prolonged periods of time in vitro. Cells are maintained in a physiologic state without many of the artifactual conditions imposed by more standard methods of tissue culture. The technique is, therefore, especially well suited for the culture of cells both of endocrine origin and their target cells. Response of cells to various hormones and their culture over long periods have been achieved thus making the techniques useful for continuous production of hormones for both laboratory and clinical uses. Modifications have been made to the basic design. These permit study of nutrient and product transport through the tissues formed in the intercapillary spaces, the effect of lymphatic-type drainage upon cell function, effects of a physiologic collagen sub-stratum upon cell growth, and study of cell physiology by nuclear magnetic resonance.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: A method is being developed for studying hormonally responsive tissues in vitro. Both mammary and ovarian tissues are target organs that are being used to determine the mechanism of action of prolactin.

Methods Employed: A bundle of tube-shaped semi-permeable membranes perfused with tissue culture medium, simulates the in vivo capillary bed. Isolated cells or tissue masses injected into the extracapillary space receive nutrients and have cell products removed by diffusion through the capillary walls. In this nearly physiologic environment, established cell lines form solid masses of tumor while primary tissue explants continue to function in a normal manner, in some cases, for several months.

Major Findings: The standard artificial capillary culture unit, which was developed in this laboratory, has previously been shown to provide a nutrient matrix which permits established cells to grow and form solid tumor masses in vitro. Secretory cells have retained their function in such a system for several months. Modification of the recirculating perfusion medium into a single pass mode of operation has demonstrated its value in studying the response of cell masses to physiologic hormones and chemotherapeutic agents.

Two separate capillary bundles were woven together and placed in a plastic shell fitted with Y connectors at each end. This provided matrices that were simultaneously intimately intertwined but still distinctly separate. Operation of the same or different hydrostatic pressures in co-current or counter-current perfusion modes permitted study of transport of solutes by diffusion or bulk flow through tissues grown within this braided network.

Configuational modifications of the encasement shell allow insertion of the capillary bundle into the head of an NMR probe and subsequent study of high density tissue growth under a variety of rigidly defined and controlled conditions. The cells currently being used are bovine papilloma virus transformed mouse C127I cells that contain the rat proinsulin gene. The cells do, therefore, synthesize and secrete proinsulin. The rates of secretion and responses to incubation conditions are being studied using the capillary culture technique.

Significance to Biomedical Research and the Program of the Institute: The characteristics of the capillary culture unit enable rapid responses to both stimuli and inhibitors of cell function to be studied in a nearly physiologic in vitro environment. This work has demonstrated that convection as well as diffusion of cell nutrients and products plays a role in the transport of various substances within tumors. This may be of assistance in pharmacokinetic modeling and in understanding and improving the transport of both physiologic and pharmacologic agents within solid tumors. The study of cell behavior using NMR techniques may now be possible and provide a unique opportunity to study tumor physiology by a non-invasive method.

Proposed Course of Research: Ovarian, mammary tissues, and hormone-producing transformed cells will be studied within the artificial capillary culture unit in conjunction with our efforts to study the growth and regression of hormonally-responsive tumors in vitro. Prostaglandin synthesis by these tissues will be

determined in response to prolactin and steroid stimulation. The ductal and alveolar structures of the rat mammary gland will be cultured on artificial capillaries coated with various types of collagen in an attempt to culture normal mammary tissue in a three dimensional structure and thus provide an *in vitro* model that more accurately reflects the *in vivo* state for the study of mammary gland growth and tumor regression. NMR studies will be made on high cell density cultures, studying the effects of hormonal, nutrient, and drug manipulations.

Publications:

1. Knazek, R.A., Gullino, P.M. and Frankel, D.S. Dual circuit woven artificial capillary bundles for cell culture: #3.4,206,015, June 3, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08210-07 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Changes Related to Onset and Cessation of Normal Cell Growth

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Herbert L. Cooper Chief, Cellular and Molecular Physiology Section — LPP, NCI

Others:

Richard Braverman	Chemist	LPP, NCI
Roberto Fagnani	Visiting Associate	LPP, NCI
Julianne Sando	Staff Fellow	LPP, NCI

COOPERATING UNITS (if any)

Laboratory of Biochemistry, Enzyme Chemistry Section, NIDR

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To characterize the behavior of ribosomal particles in a non-growing cell population in which the rate of protein synthesis is maintained at low levels; to correlate such behavior with the limitation in protein synthesis so as to understand the mode of translational control employed by the cell in adjusting protein synthesis to growth and metabolic activities; to determine the regulatory steps which are operative in the enhancement of protein synthesis associated with the onset of cell growth; to relate modifications of the cell membrane with biochemical alterations regulating nuclear and cytoplasmic functions as they participate in control of cell proliferation; to detect and characterize the biochemical steps which control protein synthesis during the physiological cessation of cell growth and division.

PHS-6040
(Rev. 2-81)

Project Description:

Major interest has been focussed on the biochemical mechanisms involved in the control of protein synthesis during growth activation in human lymphocytes. Related studies examine, (1) the biochemical events which are involved in and follow from binding by lymphocytes of substances which promote normal or neoplastic growth; and (2) unusual post-translational modifications of specific proteins during the growth cycle.

Methods Employed: Studies were performed on human peripheral lymphocytes, purified from heparinized venous blood of normal donors by combined nylon column adsorption and density separation in Ficoll-isopake. RNAs were prepared by phenol extraction and density gradient fractionation. Transfer RNAs were separated by benzoylated-DEAE column chromatography and amino-acylated with either commercial E. coli aminoacylsynthetase or rat-liver synthetase prepared in our laboratory.

Protein synthesis rates were estimated by incorporation of acid-insoluble radioactivity from [^{3}H]leucine added for pulse periods. DNA was quantitated by the Burton procedure.

Two dimensional gel analysis of proteins was performed by a modification of O'Farrell's technique with Coomassie-blue staining for total proteins and fluorography for [^{3}H]-labeled newly synthesized proteins.

Interferon preparations were: 1) an impure preparation of lymphoblastoid interferon from Burroughs-Wellcome (sp. act. 1.5×10^6 U/mg protein) and 2) a partially purified preparation given by Dr. E. Knight (E.I. Dupont) of human fibroblast interferon (sp. act. 10^7 U/mg protein).

Fractionation of cells into subcellular components was performed by nitrogen cavitation and differential centrifugation.

Translation initiation factors were prepared by heparin-sepharose chromatography of lymphocyte cytoplasm, combined with sucrose density sedimentation.

Major Findings: We have completed the first part of a study on the relationship of initiator $tRNA^{met}$ concentrations to rates of protein synthesis during lymphocyte growth. Briefly, we found that a strict linear correlation exists between the concentration of $tRNA_i^{met}$ and protein synthetic rate at all times during the lymphocyte growth cycle. This includes both the phase of transition from resting to growing state, and the period of decline from rapid growth toward the resting state again. Calculation of absolute $tRNA_i^{met}$ concentrations gave a range from 0.85×10^{-7}M (resting) to 3.1×10^{-7}M (rapidly growing). The K_m for the binding reaction between $tRNA_i^{met}$ and eIF-2 (initiation factor 2) - the first step in protein synthesis - has been reported to be 2×10^{-7}M. Therefore, the concentration of $tRNA_i^{met}$ in lymphocyte cytoplasm is in the range which may be rate-limiting for the first binding reaction, and hence for protein synthesis.

We found that $tRNA_i^{met}$ was maintained at a fixed ratio to total tRNA concentration throughout the growth cycle, implying that its increased concentration may arise from a general increase in tRNA synthesis rather than from a mechanism

specific for the initiator species. However, in a study of the relative proportions of 12 other tRNAs during growth activation, we found that those proportions changed in various ways. Thus, accepting activities for some amino acids showed a relative rise during growth induction (e.g.-glu, gly); others remained constant in proportion (e.g.-met_i, pro, val) while still others showed a relative decrease (e.g.- asp, ser, phe). These changes are relative, and occur in the context of an overall rise in absolute tRNA levels. Therefore, $tRNA_i^{met}$ levels may be regulated as part of a subset of tRNAs, rather than all tRNAs. Our evidence suggests that $tRNA_i^{met}$ concentration may play a regulatory role in protein synthesis. Changes in its concentration related to the cell growth cycle must involve control mechanisms which operate at the level of transcription and processing of tRNAs. This area will be a subject of future study.

Work is in progress on the regulatory role of initiation factor eIF-3 in controlling lymphocyte protein synthesis. This factor (a complex of 9 proteins) is required for maintaining the dissociated state of ribosomal subunits, a condition essential for their participation in protein synthesis. We have previously shown that, in resting lymphocytes, ribosomes are continually shunted into an inactive pool due to failure of dissociation. Following growth stimulation, this trend is reversed, causing activation of previously inactive ribosomes needed for increasing protein synthesis. These observations suggest that increased activity of eIF-3 is a necessary early requirement for stimulating protein synthesis during lymphocyte activation. Our current study is aimed at determining whether alterations in quantity or in distribution of the components of the eIF-3 complex are associated with the acceleration of protein synthesis during lymphocyte activation. Thus far, we have partially purified radioactive eIF-3 from human lymphocytes by heparin-sepharose chromatography and sucrose gradient density sedimentation. Activity of the preparation is determined by dissociation of 80S ribosomes prepared from lymphocytes. By two dimensional electrophoresis in polyacrylamide gels, we are attempting to determine whether differential changes in relative rates of synthesis of various components of the eIF-3 complex may occur, or whether charge modifications occur which might indicate phosphorylation or other alteration related to activation of one or more components.

The effects of interferon (IFN) treatment of human lymphocytes is being studied because of the reported activities found in IFN-treated cells which may participate in control of protein synthesis. Using 2-dimensional gel electrophoresis, we have detected 8 peptides (I-peptides), whose synthetic rates are enhanced in lymphoctes from all donors upon IFN treatment. These proteins, however, are synthesized in small amounts by untreated cells, so any activities associated with them may have physiological functions which are exaggerated by IFN. Although IFN-related activities are generally inhibitory to protein synthesis, enhanced production of these proteins does not reduce the already low rate of protein synthesis in resting lymphocytes. However, following growth stimulation, IFN causes a modest (ca. 30%) reduction in the degree of stimulation of protein synthesis. The same 8 peptides are increased by IFN in growing cells. These results may indicate that a major control of protein synthesis in resting cells preempts any inhibitory effect of IFN-related mechanisms. Relief of the primary inhibition by growth stimulation may permit detection of the less potent IFN effects. Current studies are aimed at further definition of the I-peptides in order to relate them to the functional activities reported in IFN treated cells or to relate them to activities not yet

discovered. Our approach includes electrophoretic transfer of proteins from acrylamide gels to millipore or other matrices, where various properties of separated peptides, such as binding of double-stranded RNA, may be directly measured for individual peptides. Such activity would relate the I-peptides to reported IFN-related enzymes, which are known to require double-stranded RNA for their activation. In addition, studies are in progress to localize the I-peptides to different subcellular compartments by cell fractionation, using the nitorgen cavitation technique. Initial results with this approach show that two of the most prominent I-peptides are found predominantly in the cytosol (post-ribosomal supernatant), while another is found in both the cytosol and the ribosome-endoplasmic reticulum fractions. This latter protein ("I-2") is of considerable interest, since its molecular weight - ca. 67,000 - makes it a candidate for one of the reported IFN-related activities, namely, 2'-5' A polymerase. It is of obvious importance to determine whether its particular location is ribosomal per se, or depends on the membranous component of the endoplasmic reticulum. The former possibility would be consistent with a role for this protein in the regulation of protein synthesis. By purification of the various classes of lymphocytes in the starting cell population, we have determined that IFN enhances I-peptide synthesis in the T-lymphocytes. B-lymphocytes and monocytes show little enhancement of I-peptides. Interestingly, monocytes may show enhancement of a different set of peptides.

In another area, we are investigating the interaction of a lymphocyte growth factor - T-cell growth factor (TCGF or Interleukin 2) - with lymphocytes and the biochemical consequences of this interaction. This study is being conducted by Dr. Fagnani. TCGF is absolutely required for growth of cultured T-lymphocytes after the initial few passages in culture. It is produced by the T-lymphocytes themselves upon activation by mitogens, reportedly under the stimulation by another factor - Interleukin 1, produced by macrophages in the initial culture. We have shown that resting peripheral lymphocytes do not bind TCGF. However, after mitogen activation, the stimulated lymphocytes will quantitatively remove TCGF from the culture medium. That this is a surface binding reaction is shown by the fact that we can recover virtually all of the TCGF activity, after binding, by elution at low pH. Thus, it appears that a functional binding site for TCGF is absent from resting lymphocytes but appears after growth stimulation.

An attempt was made to prepare radiolabelled TCGF by incubating mitogen-stimulated cells for 24 hrs. with [^{3}H] leucine. The culture fluids were recovered, dialysed and concentrated, then exposed to large numbers of activated lymphocytes which removed TCGF from the preparation. Upon extraction of these cells at low pH, radioactive proteins as well as TCGF were recovered. As shown by Sephadex G-200 chromatography, most of the eluted radioactive proteins eluted between M_r markers at 21000 and 10000. TCGF activity eluted slightly ahead of the peak of radioactivity, but still behind the 21000 M_r marker. Thus, the bulk of radiolabelled protein synthesized by, and subsequently bound and eluted from, growing lymphocytes, is other than TCGF. Polyacrylamide SDS-gel electrophoresis of the G-200 fractions described above revealed a minute amount of labeled protein with a single band having a M_r ca. 18000 in the fraction containing the peak of TCGF activity.

Other studies of TCGF-lymphocyte interaction include a survey of the biochemical effects of TCGF treatment of cultured T-lymphocytes (CTC). These include characterization of overall effects on RNA and protein synthesis, effects on synthesis of specific peptides, and behavior of ribosomes and polysomes during growth stimulation and cessation. Upon removal of TCGF from growing CTC, the rates of RNA and protein synthesis begin to decline almost immediately. Within 24 to 36 hrs., the cells have returned to nearly resting cell levels. Polysome profiles at this time show the pattern characteristic of resting lymphocytes (major peak of single 80S ribosomes, few polysomes). Upon replacement of TCGF, protein synthesis is stimulated almost immediately. After 2.5 hrs., the rate is nearly doubled and 2-dimensional gel electrophoresis shows that differential stimulation of a specific set of proteins occurs. These proteins are the same ones which are activated early in mitogen stimulation of peripheral lymphocytes, indicating their general importance in the onset of growth in resting cells. Further studies will include characterization of the sequential changes in specific peptide synthesis during the step-up and step-down phases, to focus on those proteins whose synthesis is critical to these changes.

Studies by Dr. Sando are beginning, in the area of the interaction of the tumor promoter, phorbol ester, with the lymphocyte. It has been shown that phorbol ester binds to lymphocytes at a specific site; that this binding modifies the response of the lymphocyte to certain mitogens, both enhancing growth response, and provoking synthesis of specific products, including TCGF. It was found that, following binding to phorbol, the bound material, together with the receptor, is rapidly lost. It was also shown that certain mitogens are directly competitive with phorbol for binding. These observations are being pursued to determine whether the phorbol-binding site complex is shed from the cell surface or internalized, and to characterize the state of the cell following this loss.

In a continuing collaboration with the enzyme chemistry section, NIDR, we have identified a novel amino acid, hypusine, which is formed in lymphocyte protein after growth stimulation. This amino acid is formed by the covalent binding of a portion of the polyamine, spermidine, with a lysine molecule in a peptide chain. We have found that this modification is restricted almost entirely to a single protein ("H"-protein) of M_r ca. 18000 and moderately acidic isoelectric point. The protein is continuously synthesized by lymphocytes in both resting and growing states. However, the hypusine modification occurs only after growth stimulation. The same protein has been detected in every other cell line studied, and seems to be a ubiquitous characteristic of growing cells. Future work in this Laboratory will aim at subcellular localization of the H-protein in an effort to gain insight into its function.

Significance to Biomedical Research and the Program of the Institute: By the elucidation of the mechanisms which regulate protein synthesis in resting cells and during a physiological growth sequence, we hope to provide the basis for recognition and understanding of derangements in these controls which may characterize malignant transformation. This objective supports Objective 3-1 of the NCP.

Proposed Course of Research: The projects described will be continued along the lines indicated in the preceding description.

Publications:

1. Cooper, H.L. and Braverman, R. Protein synthesis in resting and growth-stimulated human peripheral lymphocytes: Evidence for regulation by a non-messenger RNA. Exp. Cell Res. 127: 351-359, 1980.

2. Lester, E., Lemkin, P., Lipkin, L. and Cooper, H. Computer-assisted analysis of two-dimensional electrophoresis of human lymphoid cells. Clin. Chem. 26: 1392-1402, 1980.

3. Resch, K., Wood, T., and Cooper, H.L.: Demonstration of free dissociation factor activity in cytoplasm of lymphocytes. FEBS Lett. 117: 284-288, 1980.

4. Lester, E.P., Lemkin, P., Lipkin, L. and Cooper, H.L.: A two-dimensional electrophoretic analysis of protein synthesis in resting and growing lymphocytes in vitro. Journal of Immunology 126: 1428-1434.

5. Park, M.H., Cooper, H.L. and Folk, J.E.: Identification of hypusine predominantly in a single protein of human lymphocytes: spermidine as its precursor. Proc. Natl. Acad. Sci. U.S.A. In press, 1981.

6. Resch, K., Wood, T., Northoff, H. and Cooper, H.L.: Microtubules: are they involved in the initiation of lymphocyte activation? Eur. J. Biochem. In press, 1981.

7. Cooper, H.L. and Braverman, R.: Close correlation between initiator methionyl tRNA level and rate of protein synthesis during human lymphocyte growth cycle. J. Biol. Chem. In press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08212-07 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
From Gene to Protein: Structure, Function and Control in Eukaryotic Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.L. Berger	Research Chemist	LPP, NCI
Others:	G.P. Siegal	Research Associate	LPP, NCI
	D.M. Wallace	Visiting Fellow	LPP, NCI
	R.S. Puskas	Senior Staff Fellow	LPP, NCI
	L.A. Liotta	Expert	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Stimulation of human leukocytes in vitro with the mitogen, staphylococcal enterotoxin A, results in highly variable quantities of interferon. Of the cultures tested, approximately 30% did not produce detectable γ- or immune interferon, and about 30% synthesized significant quantities. Messenger RNA isolated from the cells of selected donors gave rise to functional γ-interferon when translated in Xenopus laevis oocytes. The mRNA coding for this protein is the largest interferon mRNA so far encountered in human cells, sedimenting at 18S in formamide-sucrose density gradients. Relative to the cytoplasmic polyadenylated RNA obtained from the entire leukocyte population, the level of γ-interferon mRNA on a molecular basis was approximately ten parts per million.

Project Description:

Major Findings: Upon stimulation with antigens or mitogens, cultured lymphocytes isolated from the peripheral circulation of normal donors produce human γ-interferon also known as immune or T-interferon. This class of interferon differs markedly from both leukocyte and fibroblast interferons. It is never virally induced and therefore may not function primarily as an antiviral substance in vivo. Immune interferon has not been purified to homogeneity but it can be distinguished from leukocyte and fibroblast interferon either antigenically or by its instability at pH 2. Compared with other interferons it is a large protein; estimates of its molecular weight range from 40,000 to 70,000. Preliminary evidence suggests that immune interferon, itself, may be heterogeneous.

The production of immune interferon by human leukocytes has been characterized. Cells purified from plateletpheresis residues by Ficoll-Hypaque gradient centrifugation and cultured in vitro in heterologous human serum were stimulated with a variety of mitogens. The accumulation of immune interferon in the culture fluids and in some cases the rate of DNA synthesis were determined for seven days following activation. Routine methods were used throughout. When staphylococcal enterotoxin A (a gift of Dr. Leonard Spero) was used as the mitogen, only 70% of the cultures studied produced γ-interferon. Of those, only 40% produced sizable quantities. Maximum levels of immune interferon were achieved in capable cultures between 3 and 6 days after activation. In contrast, cultures that had produced no interferon by day 3 did not synthesize it thereafter. In some cases the defect could be traced to the serum but the nature of the defect has not yet been determined. When autologous serum was tested, immune interferon synthesis by the leukocyte population plummeted relative to the levels achieved by the same cells in a variety of heterologous sera. Comparison of interferon yields with the degree of transformation of the cells measured by radioactive thymidine incorporation into DNA suggested dissociation of the two events. It was not possible to predict interferon yields from the rate of DNA synthesis. However, in the absence of a mitogen or in the absence of serum, immune interferon production was never observed.

Several mitogens were evaluated for their ability to induce immune interferon synthesis in cultured leukocytes. In general, staphylococcal enterotoxin A treatment resulted in the highest levels of interferon; on occasions, staphylococcal enterotoxin B was equally effective, as was phytohemagglutinin. The time at which the peak levels occurred varied with the mitogen and also with the donor. Concanavalin A and exfoliatin were inevitably poorer inducers of interferon when compared under identical conditions of cells and sera. It must be emphasized, however, that any single culture might deviate radically from these norms. Immune interferon production in mixed leukocyte cultures was also studied in the presence and absence of staphylococcal enterotoxin A. Lacking the mitogen, mixed cultures produced immune interferon after 5-6 days. In the presence of staphylococcal enterotoxin A, cells from two different donors each of which synthesized copious amounts of γ-interferon when cultured separately, produced virtually no interferon when cocultured with the mitogen. These results serve to emphasize the complexity of the lymphocyte response to external agents.

Messenger RNA was isolated from stimulated leukocytes either 48 or 72 hours after induction with staphylococcal enterotoxin A. Of necessity, the preparation of

RNA anticeded the results of the assay for immune interferon in the culture supernatants. Subsequently RNA from selected cultures with high titers of interferon (>100 Units ml^{-1}) was pooled in order to avoid dilution of immune interferon mRNA from nonproductive donors. The RNA was prepared from cytoplasm by methods which are routine in this laboratory. They make use of ribonucleoside-vanadyl complexes as nuclease inhibitors. Polyadenylated RNA was obtained from the pooled material and fractionated in sucrose density gradients. When the fractions were microinjected into the oocytes of Xenopus laevis, only the mRNA sedimenting at 18S gave rise to interferon. Furthermore, interferon was secreted, suggesting that a glycosylated precursor was synthesized and processed by the frog oocyte in response to the human mRNA. These interferon mRNA molecules were clearly larger than any of the mRNAs coding for either leukocyte or fibroblast interferon. They are therefore unique in the interferon system.

Further characterization of the interferon produced by oocytes in response to the 18S mRNA from staphylococcal enterotoxin A was carried out either by treating the samples with specific antibodies or by incubating them at pH 2. The putative immune interferon was neutralized neither by antibodies to leukocyte interferon nor by antibodies to fibroblast interferon. In addition, the antiviral activity of the interferon was completely destroyed by acid treatment. Although none of these tests represents a positive identification, they rule out all other known interferons. The conclusion was drawn that an 18S mRNA indeed coded for immune interferon.

The frequency of immune interferons among bulk leukocyte mRNAs was also estimated. By measuring the interferon synthesized by oocytes injected with a known quantity of unfractionated mRNA, it was possible to deduce the interferon mRNA levels of selected mitogen-induced cultures relative to those of unselected virus-treated cultures. In the former case, the cells synthesized γ-interferon; in the latter, leukocyte interferon virtually exclusively. The immune interferon levels were approximately 1-2% of the leukocyte interferon titers after normalization. Such a comparison of the two human interferon systems depends on two assumptions: (i) the efficiency of translation of immune interferon mRNA is equivalent to that of leukocyte interferon mRNA in the oocyte system; (ii) the specific activities of the two antiviral proteins are comparable. The relative amounts of the two interferon mRNAs can be converted into absolute quantities provided the frequency of interferon mRNA is known for one of them. Since Weissmann and colleagues have shown that 0.1% of the polyadenylated RNA obtained from virally-induced leukocyte cultures is leukocyte interferon mRNA, it follows, given these assumptions, that the level of immune interferon mRNA in cultures from selected donors treated with staphylococcal enterotoxin A is approximately 10 parts per million on a molecular basis. Regardless of which cells in the population produce γ-interferon, the molecular cloning of its mRNA from these preparations using the same methods which were successful for leukocyte interferon would, on statistical grounds, require screening 100-fold more clones.

The effect of interferon on lymphocytes has also been studied using the enzyme 2-5A synthetase as a marker for an interferon-treated cell. This enzyme is believed to be induced by interferon and activated by double stranded RNA. The enzyme produces a 2'-5'-linked oligoadenylate compound which, in turn, activates a nuclease

responsible for degrading the invading viral RNA, among others. Our preliminary data indicate that 2-5A synthetase levels are not necessarily related to interferon mediated phenomena in normal lymphocytes. The project has been discontinued owing to the highly variable nature of the results.

The interferons produced by lymphocytes are now being tested for their ability to inhibit growth and metastatic ability of malignant cells. Although these studies are as yet incomplete, preliminary data suggest that the action of interferons is transient and complicated depending upon the cell type tested.

Significance to Biomedical Research and the Program of the Institute: This project conforms to the Objective #3, Approach #1 of the National Cancer Plan. Its aims at an understanding of the biochemical control mechanisms by which normal cell growth and function are maintained. Disordered cell growth in neoplastic populations may then be better understood and rational attempts made to prevent or modify it.

The project also conforms to Objective #2, Approach #2. Since interferon produced in vivo or administered as an external agent may be effective in preventing or curing some types of cancer, it is essential to understand both its modes of production and its mechanism of action in normal, virus-treated and malignant cells.

Proposed Course of Research: The mechanism of action of reverse transcription is being investigated. At present extremely low yields of cDNA produced with this enzyme hamper studies of gene expression of rare mRNAs. The effect of ribonucleoside-vanadyl complexes on many enzymes will be determined in order to extend their usefulness to reactions of interest to molecular biology. Long term objectives include studies of the gene expression of rare messenger RNAs in resting and activated lymphocytes. Molecular cloning methods will be employed.

Publications:

1. Berger, S.L., Wallace, D.M., Siegal, G.P., Hitchcock, M.J.M., Birkenmeier, C.S. and Reber, S.B. Preparation of interferon messenger RNAs with the use of ribonucleoside-vanadyl complexes. In Pestka, S. (Ed.): Methods in Enzymology, 79B. New York, Academic Press, 1981.

2. Wallace, D.M., Hitchcock, M.J.M, Reber, S.B. and Berger, S.L. Translation of Human Immune Interferon Messenger RNA in Xenopus laevis oocytes. Biochem. Biophys. Res. Commun. 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08215-04 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of the Angiogenesis Factor(s)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Raju	Staff Fellow	LPP, NCI
Other:	P.M. Gullino	Chief, LPP	LPP, NCI
	M. Ziche	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Since copper appears to be an indispensable component of the angiogenesis process, ceruloplasmin, the copper-carrier in serum, was treated with trypsin and fragments of the molecule were isolated. Their angiogeneic capacity was tested and correlated with their content of copper. The experiments are not complete at this writing.

PHS-6040
(Rev. 2-81)

Objective: To elucidate the mechanism of angiogenesis.

Project Description:

Methods: (1) Ceruloplasmin containing different amounts of copper ions was isolated by ion exchange chromatography. (2) Ceruloplasmin was fragmented by tryptic digestion and the tryptic fragments were separated by gel filtration techniques. (3) Angiogenic capacity of each fragment was tested with the corneal angiogenesis test.

Major Findings: (1) Angiogenic capacity is localized in ceruloplasmin fragments containing copper. (2) A fragment smaller than 1500 MW appears to show the highest angiogenic capcity at the lowest dose.

Significance to Biomedical Research and the Program of the Institute: Isolation of a ceruloplasmin fragment able to induce angiogenesis in nanogram quantities may permit the development of a RIA to be applicable to biopsy tissue. Previous work showed that angiogenic capacity is acquired by cell populations at high risk of neoplastic transformation. A RIA able to demonstrate concentration of components of the angiogenesis process in a tissue may predict high risk of neoplastic transformation.

Proposed Course of Research: To complete the project.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08218-06 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Studies on Hormonal Mechanisms Regulating the Expression of Milk Proteins in Rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.K. Qasba	Expert	LPP,NCI
Others:	A. Dandekar	Visiting Fellow	LPP,NCI
	T. Horn	Staff Fellow	LPP,NCI
	E. Devinoy	Visiting Fellow	LPP,NCI
	M. Siegel	Biologist	LPP,NCI
	B. Stubblefield	Biologist	LPP,NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.8	4.0	0.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

For the studies of the expression of milk proteins during the functional development of the rat mammry gland and in mammary tumor MTW9, the cDNA sequences complementary to milk protein mRNAs were cloned in E. coli cells and the clones characterized. The DNA sequence of α-LA cDNA clone analysis shows that rat α-LA has 17 extra residues beyond the C-terminus of the α-lactalbumin isolated and sequenced to date from other species. The predicted C-terminal sequence is hydrophobic, proline rich and bears some resemblance to β-casein sequences. Additional results show that 1) more than one plasmid DNA with differences in the restriction maps have been identified for several of the milk proteins except for α-LA, suggesting either a presence of a family of genes or allelic differences for these proteins; 2) the expression of individual milk proteins is dependent on the functional stage of the gland; 3) there is an inverse relationship between the expression of milk proteins and the methylation of the gene sequences; 4) mammotrophic hormones required for synthesis and stability of milk proteins and their mRNAs, when withdrawn arrest the synthesis α-LA in the mammary tumor MTW9 at 6 h or earlier of withdrawal but without any measurable effect on other proteins of the tumor.

Project Description:

Objectives: The purpose of this work is to study the synthesis of α-lactalbumin, casein proteins and other milk proteins and their mRNAs in mammary gland at different stages of functional differentiation of the gland and in mammary tumors. The studies have been focused on: a) construction and characterization of the E. coli plasmids containing α-lactalbumin and casein cDNA sequences; b) organization and state of methylation of α-LA and casein structural gene sequences in the DNA from rat mammary gland at different stages of functional development and from mammary tumors, and c) analysis of in vitro synthesized translation products directed with the Poly A^+ RNA isolated from (1) mammary gland at different stages of functional development and (2) from mammary tumors.

Methods Employed: 1. Double stranded cDNA was synthesized by use of viral reverse transcriptase from the poly A^+ RNA fractions enriched for either α-LA mRNA or casein mRNA. The ds-cDNA was inserted in the Pst 1 site of BR322 by using the oligo(dc).oligo(dg) joining procedure. After bacterial transformation colonies carrying α-LA DNA or casein DNA were identified by positive hybrid-selection procedure. 2. The methods of colony hybridization, DNA/ RNA transfers, DNA, RNA and protein electrophoresis, isolation and in vitro translations of RNA etc., has been detailed in project Nos. Z01 CB08231, and 08219.

Major Findings:

cDNA clones for low molecular weight lactoproteins. Three classes of cDNA clones containing gene sequences for 21-22.5K daltons rat lactoproteins have been identified. Plasmid DNA of p-α-LA clones (class I) have rat α-LA structural gene sequences. The DNA sequence analysis shows that rat α-LA has 17 extra residues beyond the C-terminus of the α-lactalbumin isolated and sequenced to date from other species. The predicted C-terminal sequence is hydrophobic, proline rich and bears some resemblance to β-casein sequences. Plasmid DNA of p-k clones (class II) have structural gene sequences for an, as yet, unknown protein in rat milk which is devoid of cysteine. Plasmid DNA of p-Wp clones (class III) have gene sequences possibly for rat whey phosphoproteins. The plasmid DNAs belonging to each of these classes have unique restriction maps, are specific to mammary gland and hybridize to specific mRNA species.

cDNA clones for rat caseins. Double stranded cDNAs synthesized from poly(A)-RNA enriched for rat casein mRNA sequences were cloned in E.coli after insertion at the Pst I site of pBR322. Recombinant plasmid DNAs carrying structural gene sequences for caseins have been correlated with their corresponding rat milk caseins. Two types of clones for 42K and 25K caseins have been identified which differ in their restriction maps.

Plasmid p-X 305 (850 bp insert) when nick-translated and used in Northern blot analysis of poly(A)-RNA, hybridizes to a mRNA region of the gel encoding synthesis of 2 proteins in vitro. One of the proteins is immunologicaly similar to the 29K milk casein and the other has an apparent molecular weight of 22K daltons. However, when p-X 305 DNA is used in the hybrid selection assay, it specifically retains mRNA encoding the 22K dalton 'X' protein. This 22K protein was precipitable by casein antiserum, but could not be competed out by any of the known rat milk caseins--42K, 29K, or 25K proteins or by any other known rat milk

proteins namely α-LA and whey phosphoproteins (Wp_1, Wp_2, and Wp_3). The cDNA clone for 29K casein remains to be identified.

Unique restriction sites for each of the cDNA clones. cDNA clones that contain sequences which correspond to mRNAs of 6 different milk proteins have been characterized by restriction analysis. Initial grouping of these clones was based on the positive hybrid-selection translation assay. All 6 different classes of clones have unique restriction patterns which make them quite distinct from each other.

p-α-LA clones have Xba I site which is not present in any other class. It also has Msp I and Sst I sites which are adjacent to each other, followed by an Eco RI site 30 nucleotides towards 3' end, before the Xba I site (table 1).

p-k clones contain a Kpn I site not present in any other class. This Kpn I site is between Hha I and Pvu II site. The distance between the Pvu II and Kpn I sites is the feature that distinguishes the two types of clones in this category. p-k 94 has about 110 nucleotides more (shown as a loop) as compared to p-k 70.

p-Wp clones are of two types: p-Wp 47 and p-Wp 52. These clones have two Bam HI sites and one Hind II site. Two features distinguish the two types of clones: 1) the placement of the two Hink I sites and 2) hybridization to p-k 70 DNA, p-Wp 47 hybridizes but not p-Wp 52.

'X'-protein cDNA clone corresponds to the unknown casein ('X'), has a Sst I but no Eco RI or Xba I site.

p-25K clones have a Pst I site not found in the other 5 classes of clones. There are two types: p-25K 530 with a Bgl II site and p-25K 101 with an Eco RI site.

p-42 clones have two Hind III sites not present in other class of clones. There are two types: p-42K 303 and p-42K 53. The two Hind III sites are more apart in p-42K 303 than in p-42K 53 and the latter has a single Bam HI site between the two Hind III sites.

For four of the rat lactoproteins there are two types of cDNA clones, suggesting either allelic differences or a family of genes for these proteins. The differences between the two types, in most cases are not single base pair alterations and since many individual clones belonging to each type were analyzed, these differences are less likely to be due to an artifact of cloning procedure.

Gene expression in normal rat mammary gland during functional differentiation of the gland and in rat mammary tumors. In continuation of our previous work (Nakhasi and Qasba, J.Biol.Chem. 254: 6016, 1979), we have translated the poly A^+ RNA from the mammary gland at reproductive stages and analyzed the translational products and their immunoprecipitates on SDS-gels. We have found that resting, pregnant and lactating mammary gland RNAs encode characteristic translation products. Various classes of RNAs have been distinguished. Some are present only in virgin mammary gland, others are present at all stages. Some RNAs, especially the major milk proteins, increase during pregnancy, while others apparently

disappear during lactation. The sequences corresponding to each clone, measured by "dot blot" analysis, increased during the functional differential of the gland, but in a pattern which was unique to each m-RNA sequence. (see T. Horn's project report). Several rat mammary tumors show the expression of only one or the other species of milk proteins. This pattern is unique to the tumor and is different as compared to normal rat mammary gland pattern at any given stage of functional differentiation.

Significance to Biomedical Research and the Program of the Institute: Understanding of the normal development and differentiation of mammary gland and the alterations which have occurred in tumors, will help in designing experiments for early detection and appropriate treatment of breast cancer.

Proposed Course of Research: The experiments will be further carried out to study: 1. The organization α-LA and casein gene sequences in rat mammary gland at different stages of development and in mammary tumors. 2. The altered expression of these genes in mammary tumors.

Publications:

1. Qasba, P.K., Dandekar, A.M., Sobiech, K.A., Nakhasi, H.L., Devinoy, E., Horn, T., Losconczy, I., and Siegel, M. Milk protein gene expression in the rat mammary gland. Critical Reviews of Food Sciences and Nutrition, 1980.

2. Dandekar, A.M. and Qasba, P.K. Rat-α-lactalbumin has 17-residue long c-terminal hydrophobic extension as judged by the sequence analysis of the cDNA clones, Proc. Nat. Acad. Sci. USA (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08223-05 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Estrogen Binding Studies in Ovarian Dependent and Independent Rat Mammary Carcinoma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. S. Bodwin	Senior Surgeon	LPP, NCI
Other:	Y.S. Cho-Chung	Research Chemist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study continues to investigate how the specific biochemical phenomenon of estrogen receptor binding is related to the cAMP binding proteins during growth and repression of ovarian dependent and independent tumors.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: 1. To characterize and measure estrogen receptors and cAMP-binding proteins in the cytosol and nucleus of ovarian dependent and independent tumors. 2. To study in vivo and in vitro, the specific relationship of the estrogen receptor to the cAMP holoenzyme during nuclear transaction and subsequent nuclear protein phosphorylation.

Methods Employed: 1. 7,12 DMBA, NMU, MTW9 with M_tTWID, DMBA #1 and MT13762 were employed as animal models. They comprise a group of ovarian independent as well as transplantable. 2. In vitro radionuclide assays of binding components were employed. 3. Sucrose density gradient analyses of receptor components. 4. Cell free recombinative studies of cytosol and puirified nuclei.

Major Findings: 1. A reciprocal relationship exists between the concentration of estrogen receptor and cAMP binding proteins during growth and regression of ovarian dependent mammary tumors. 2. This reciprocal relationship was not seen in ovarian independent tumors. 3. This reciprocal relationship was observed during tumor induced regression following ovariectomy of the host, or host treatment with DBcAMP, tamoxifen or pharmocologic doses of estradiol. 4. The relationship of estrogen receptor to cAMP binding protein appears also to be antagonistic in that each receptor will inhibit the nuclear translation of the other. This inhibition was found to be dependent upon cAMP and estradiol concentrations. 5. The phosphatase inhibitor, sodium molybdate will inhibit the nuclear uptake of the estrogen receptor. 6. ATP, arginine and Ca^{++} increase the uptake of the cAMP binding protein.

Significance to Biomedical Research and the Program of the Institute: The understanding of the biological significance of the antagonistic relationship between the estrogen receptor and cAMP holoenzyme will better elucidate the cellular biochemistry of normal and neoplastic hormone responsive cells which may lead to clinical applications for the treatment of endocrine neoplasics.

Proposed Course of Research: 1. To investigate the role of cyclic nucleotide dependent or independent phosphorylation of cytosol and nuclear binding proteins as a mechanism for the estrogen recaeptor and cAMP-binding protein antagonism during nuclear translocation. 2. To study how the ultimate interactive of hormones during tumor growth and regression may affect nuclear protein phosphorylation.

Publications:

1. Bodwin, J.S., Cho-Chung, Y.S., Hirayama, P. and Rego, J. Interactions of cAMP-binding proteins and estrogen receptors in hormone-dependent mammary tumor regressions due to tamoxifen or pharmacological doses of estrogen. JNCI, vol. 66: 321-326, 1981.

2. Cho-Chung, Y.S., Clair, T., Bodwin, J.S. and Hill, D. L'arginine and DBcAMP synergistically inhibit in vivo growth of hormone-dependent mammary tumors. BBRC 95: 1306-1313, 1980.

3. Bodwin, J.S., Hirayama, P. and Cho-Chung, Y.S. Nuclear Translocation of Estrogen Receptors and cAMP-binding protein in MTW9 Tumors. JNCI, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08225-06 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Membrane of the Mammary Gland and Dynamics under Normal and Pathological Conditions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Pinto da Silva	Chief, Membrane Biology Section	LPP, NCI
Others:	C. Parkison	Chemist	LPP, NCI
	B. Kachar	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)
Dr. Ian Mather, Department of Animal Science, Univ. of Maryland, College Park, Maryland, and Dr. A. Peixoto de Menezes, School of Medicine, University of Lisbon, Portugal

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Comparative morphology of the plasma membranes from lactating tissues and mammary carcinomas. Structural transformation of the membrane envelope of the milk fat globule after secretion and during isolation procedures. Localization of membrane proteins of the membranes of mammary gland epithelial cells. Membrane structure and dynamics of the basal region of the secretory cell and the epithelial-myoepithelial cell interactions are followed during pregnancy and lactation.

PHS-6040
(Rev. 2-81)

Project Description:

Methods: Tissue (lactating rat mammary gland, DMBA and NMU mammary carcinomas) and membrane preparations (milk fat globule membrane - MFGM) are fixed in glutaraldehyde, impregnated with glycerol, and rapidly frozen in the liquid phase of partially solidified Freon 22 at liquid nitrogen temperature. Specimens were fractured and platinum-carbon replicated in a Balzers freeze-etch device or prepared for conventional thin sectioning. All specimens were observed by TEM in a JEOL 100C EM. Monoclonal antibodies are prepared against milk fat globule membrane proteins and the plasma membrane of mammary gland acinar cells. Ferritin conjugates are prepared and mammary gland acinar cells and membrane fractions labelled.

Major Findings: Project temporarily interrupted pending isolation of monoclonal antibodies against milk fat globule membrane proteins by Dr. Ian Mather.

Significance to Biomedical Research and the Program of the Institute: There is considerable evidence of the interrelationships between the secretory process and the malignant transformations of the mammary epithelial cells. All these events are related with development of a system of cell membranes; the membrane envelope of the milk fat globules carries antigen common to the cell membrane of mammary carcinoma cells; there are common hormonal stimuli involved in the lactogenesis and in the malignant transformation. Cell membranes are also involved in other events related with tumor invasiveness and metastization. Milk fat globule membrane vesicles may be used as a natural liposome system to carry drugs to specific organs.

Proposed Course of Research: Dr. Ian Mather will attempt to obtain monoclonal antibodies against specific proteins of the milk fat globule membrane. We plan to use these antibodies to locate by ferritin and coleoidal gold conjugates the site of these proteins on the membrane of milk fat globules as well as their intracellular pathway towards secretion. More distantly, we plan to use the "fracture-label" techniques developed in our laboratory to locate prolactin receptors as well as casein molecules and its precursors.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08226-05 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
The Role of Thyroid Hormones in Growth and Differentiation of Mouse Mammary Glands

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.K. Vonderhaar	Expert	LPP, NCI
Others:	D. Liscia	Visiting Fellow	LPP, NCI
	M. Bhattacharjee	Visiting Fellow	LPP, NCI
	A.E. Greco	Chemist	LPP, NCI
	T. Alhadi	Biologist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiology

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.25	2.5	1.75

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is designed to understand the role of hormones (particularly T_3 and prolactin) in normal mammary gland development and differentiation. We wish to understand how milk-protein production is controlled by various hormones. Studies include 1) Evaluation of the role of DNA synthesis in the response of tissue after explant culture in the presence of various hormones; 2) examination of the nature of the interaction of prolactin and human growth hormone with native as well as cryptic forms of lactogenic receptors; 3) to partially purify the lactogenic receptor in order to produce monoclonal antibodies against it; 4) purify α-lactalbumin from mouse mammary glands and prepare an antibody, and 5) examine the role of thyroid hormones in synthesis and secretion of milk proteins in organ culture.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The purpose of these studies is to investigate the role of prolactin - thyroid hormone interactions in mammary gland development differentiation and tumorigenesis. The role of thyroid hormones in milk-protein production is emphasized. The coupling of milk-protein synthesis with prior DNA synthesis in virgin mouse mammary glands in vitro is to be investigated. This coupling is contrasted with lack of requirement for DNA synthesis in primiparous involuted tissue. The nature of the interaction of lactogenic hormones with their receptors, the stability of the hormone-receptor complex, and the nature of cryptic binding sites was examined. Hormonal control of cryptic sites was investigated with emphasis on thyroid hormones. Partial purification of the lactogenic receptors was attempted in order to prepare a monoclonal antibody.

Methods Employed: C3H/HeN mice were used. Mild hypothyroidism was induced by addition of 0.1% thiouracil to the drinking water. Hyperthyroidism was induced by addition of T_4 to the drinking water. Specific binding of prolactin or human growth hormone to membrane bound or detergent solubilized receptors was studied by standard competition binding techniques. Purification of α-lactalbumin and of lactogenic receptors was accomplished by using standard chromatographic and separation techniques. Organ culture was performed using chemically defined serum-free medium supplemented with various hormones and metabolic inhibitors. Production of α-lactalbumin and casein was determined by radio-precipitation assays or by immunohistochemistry (in collaboration with Dr. G.H. Smith) using mono-specific antibodies prepared against the milk-proteins.

Major Findings: Mouse α-lactalbumin was purified to homogeneity from mammary glands of lactating mice. In brief, this involved homogenization of the tissue and preparation of a post-mitochondrial supernatant. After removal of the lipid layer and sonication, $(NH_4)_2SO_4$ precipitation was performed in the presence of Mn^{++}. The α-lactalbumin was recovered in the 50-75% saturation pellet. The recovered proteins were then passed through a Bio-gel P-100 column. Active α-lactalbumin was recovered by precipitation with $(NH_4)_2SO_4$ at 75% saturation. The pellet was resuspended and after dialysis, the proteins run through a Bio-gel P-30 column followed by chromatography on DEAE-Sephacel. The active α-lactalbumin was eluted with a gradient of 20mM-500mM Tris pH 7.8. This elution resulted in a single protein peak with two distinct peaks of enzyme activity. The two peaks were collected separately and their molecular weights determined with 12% SDS-PAGE. An estimated molecular weight of 14,500 was obtained for both α-lactalbumins. 2-D gels run by Dr. Toby Horn showed 2 spots with the same molecular weight but distinct pIs.

From these observations we have concluded that mouse α-lactalbumin exists in 2 distinct charged forms. Both forms have equal activity in the lactose synthetase assay system. The charged forms probably are the result of different amounts or types of carbohydrate residues.

A mixture of the two forms of α-lactalbumin was treated with glutaraldehyde and injected subcutaneously at multiple sites in rabbits to produce antibody. The antisera obtained was tested by the Ouchterlony double diffusion technique and gave a distint sharp band with the purified antigen. No cross-reactivity was seen with mouse caseins or mouse serum proteins.

Using this antibody and one developed previously against mouse caseins, we examined hormonal regulation of milk protein production in organ culture and the coupling of this process to DNA synthesis.

Mid-pregnancy mammary tissue was cultured in the presence of insulin (I), hydrocortisone (F) and prolactin (PRL) in the presence and absence of thyroid hormones (T_3). T_3 had little or no effect on casein synthesis when cultured tissue was examined after 48 hr. A 2-3 fold increase in α-lactalbumin was found in the tissue at this time. The media was examined for effects of the hormones on secretion of milk proteins. A low level of casein was found in media both in the presence and absence of T_3, but thyroid hormone did not enhance secretion of these peptides. α-lactalbumin was found in the medium to a significant extent with T_3 enhancing secretion 3-4 fold. SDS-PAGE of the α-lactalbumin produced showed a single peak from tissue cultured in the presence of IFPRL. In the presence of T_3 two distinct peaks were seen. Only a single peak was secreted into the medium even in the presence of T_3. Thus, we concluded that T_3 selectively enhances synthesis and secretion of α-lactalbumin.

Coupling of milk-protein production to DNA synthesis in organ culture was examined using tissue from mature virgin or 9 week involuted primiparous mice. When DNA synthesis is blocked by araC, virgin tissue is unable to produce enhanced levels of casein and α-lactalbumin in response to IFPRL. Total protein and RNA synthesis is not inhibited by araC. Immunoperoxidase localization of casein in cultured explants shows that at zero time (0-5 hr in culture) 3.4% of virgin epithelial cells are positive for casein. After 72 hr in IFPRL 23.6% of the cells are positive. However, when DNA synthesis is blocked, only 1.9% of the cells have casein. In contrast primiparous tissue has 6.5% positive cells initially. After 72 in IFPRL 39.0% are positive and 38.6% are positive even without DNA synthesis.

Autoradiography of explants labelled with ^{3}H-TdR shows that in virgin explants cultured for 72 hr in IFPRL 55% of nuclei were labeled while only 2.6% of the nuclei were labeled in IFPRL and ara C. When colchicine was present during the entire culture period of 72 hr 54% of the nuclei were labeled with ^{3}H-TdR, however, only 5.6% of the nuclei were trapped in metaphase. Thus only a small portion (6%) of the cells were undergoing actual cell division compared to the 24% which became positive for casein. Therefore, we concluded that the DNA synthesis requirement for terminal differentiation of virgin mouse mammary tissue may not be related to metosis.

In collaborative efforts with Dr. W.R. Kidwell's laboratory, a role of basement membrane collagen in mammary growth has been established. This work has involved the use of cis-hydroxyproline (CHP) an inhibitor of collagen production. In an attempt to assess what effects CHP has on normal mammary gland differentiation, this drug was added to virgin mouse mammary explants in the absence of hormones or in the presence of I or IFPRL. In medium containing 10 µg proline/ml, CHP (25 µg /ml) has no effect on total protein synthesis as measured by incorporation of ^{3}H-lysine into acid precipitable material. DNA synthesis is inhibited 10% in the absence of hormones, but by 75-80% in the presence of I or IFPRL. Preliminary experiments have shown a similar inhibition of casein production in the presence of CHP.

Previously we have shown that thyroid hormones act in part by inducing lactogenic receptors in mammary glands. This induction does not require protein synthesis in vitro. Therefore, we wished to analyze what role altered thyroid status may play in regulation of cryptic binding sites for lactogenic hormones. Cryptic sites for lactogenic hormones can be demonstrated either by solubilizing membranes to release the hidden sites or by methylation of membrane phospholipids in situ. This latter reaction involves membrane bound transmethylase enzymes which utilize S-adenosyl-L-methionine (SAM) as a methyl donor and convert phosphatidylethanolamine (PE) to phosphatidylcholine (PC). Using ^{3}H-SAM, methylation of phospholipids was examined in liver and mammary gland membranes under conditions which result in opening of cryptic binding sites. 85-90% of the label was found in phospholipids after a 20 min. reaction. Of this label 65-70% is in PC with the remaining 30-35% distributed in the intermediates, phosphatidyl mono- and dimethyl- ethanolamine.

Liver microsomal membranes were prepared from hypo- hyper and euthyroid virgin mice and the phospholipid methylation and induction of cryptic binding sites examined. All three types of tissue showed similar methylation reactions; the level of the enzyme was not significantly different. Induction of cryptic binding sites was seen at 250μM SAM in all 3 cases. However, euthyroid and hyperthyroid mouse livers had 50-60% increase in binding (cryptic sites) in the presence of SAM. Livers from hypothyroid animals showed a 95% increase in binding. This latter level of cryptic sites is similar to that seen in late pregnant or early lactating mice. Thus, we concluded that while hypothyroidism reduces the total amount of binding to membrane bound receptors (previously reported), there are more cryptic sites in the hypothyroid state than in euthyroid or hyperthyroid.

In an attempt to show that the increase in binding seen on solubilization or SAM treatment is the result of unmasking of cryptic sites rather than dissociation of endogenously bound hormone and re-binding, the stability of the hormone-receptor complex was examined after solubilization or SAM treatment. Receptors were prebound with ^{125}I- oPRL or ^{125}I- hGH. Following solubilization with Triton-X100, the hormone receptor complex was allowed to incubate for up to 92 hr at room temperature. During this time 95% of the hormone-receptor complex could be recovered by PEG precipitation. Similarly, pre-bound ^{125}I-hormone receptor complex was over 80% stable for 3 days in the presence of SAM. These data point out the great stability of the lactogenic receptor when it is bound to hormone.

The lactogenic receptor is also very stable to pH and high salt concentrations. At NaCl concentrations from 0.1 to 1M, specific binding of ^{125}I-hormone to membrane bound receptors was examined. AT 1M NaCl, the specific binding is 75% of the level seen in the absence of NaCl. Non-specific binding is only 55% of the level seen in the absence of NaCl. At 0.5M NaCl no significant change in specific binding is seen while non-specific binding has dropped 30%. Solubilized lactogenic receptors are stable from pH 6 to 9 for at least 24 hr at 4°. When the proteins exposed to these pH's are returned to neutrality and tested for binding, no activity was lost. When the binding reaction was run at various pH's, however, a broad peak was seen between pH 7 and 8 with 80-85% of the activity lost at pH 9.

We utilized this stability in our attempts to partially purify the receptor from livers of lactating mice. Solubilized membrane proteins were chromatographed on a DEAE-Sephacel column. Generally a single sharp peak of PRL-receptor activity was eluted near a concentration of 0.1M NaCl. Occasionaly we saw two peaks. This may reflect different amounts of sialic acid or other carbohydrate residues on the receptor protein. The active fractions from the DEAE-column were then concentrated and placed on a Sepharose 6B column. This column separates the receptor-detergent complex on the basis of size. The receptor eluted from the Sepharose - 6B column as a single peak of activity displaced slightly from the bulk of proteins. Thus a nearly 4000 fold purification has been achieved. Following affinity chromatography on Con A-Sepharose or PRL-Affigel column the receptor will be injected into rats and a monoclonal antibody prepared.

Significance to Biomedical Research and the Program of the Institute: Prolactin-thyroid interactions are important in growth and differentiation of mammary glands. Altered thyroid status may be implicated in changes in risk of human breast cancer. Our studies are aimed at understanding whether changes in thyroid status play a direct or indirect role in mammary tumorigenesis. Thyroid hormones are known to regulate prolactin action through control of lactogenic receptors. Therefore, all aspects of prolactin binding and control of exposed as well as cryptic sites must be examined. Preparation of monoclonal antibodies against the lactogenic receptors will readily allow us to study the receptor molecule itself in the absence of the hormone and thus determine if lack of prolactin binding in certain mammary tumors is due to lack of receptors or only their existence in masked forms.

Proposed Course of Research: We will continue to study control of lactogenic receptors by thyroid hormones. Work will involve studies on membrane composition and fluidity. Phospholipid methylation in various thyroid states and in the presence of T_3 in culture will be examined. Transplantation of mammary fragments from animals in various thyroid states will be performed and subsequent tumor incidence examined. These studies are aimed at determining whether the effects of altered thyroid status are irreversible. Work will continue on the inhibition of collagen synthesis and its effects on milk-protein production. The effects of thyroid hormones on secretion of milk-proteins and glycosylation of α-lactalbumin will be examined. A monoclonal antibody against lactogenic receptors will be prepared.

Publications:

1. Wicha, M.S., Liotta, L.A., Vonderhaar, B.K. and Kidwell, W.R. Effects of inhibition of basement membrane collagen deposition on proliferating rat mammary epithelium. Developmental Biology 80: 253-266, 1980.

2. Vonderhaar, B.K. Effect of thyroid hormones on mammary tumor induction and growth. In Hormonal Regulation of Experimental Mammary Tumors. B.S. Leung (ed.), Pergamon Press, Inc. (in press).

3. Alabaster, O., Vonderhaar, B.K. and Shafie, S.M. Metabolic modification by insulin enhances methotrexate cytotoxicity in MCF-7 human breast cancer cells. Europ. J. of Cancer (in press).

4. Lewko, W.M., Liotta, L.A., Wicha, M.S., Vonderhaar, B.K. and Kidwell, W.R. Sensitivitry of N-nitroso-methylurea-induced rat mammary tumors to cis-hydroxyproline, an inhibitor of collagen production. Cancer Res. (in press).

5. Alhadi, T. and Vonderhaar, B.K. Induction of lactogenic hormone binding in livers of adult female mice treated neonatally with estradiol or nafoxidine. Endocrinology (in press).

6. Bhattacharya, A. and Vonderhaar, B.K. Membrane modification differentially affects the binding of the two lactogenic hormones--human growth hormone and ovine prolactin. Proc. Natl. Acad. Sci. USA (in press).

7. Kidwell, W.R., Knazek, R.A., Vonderhaar, B.K. and Losconczy, I. Effects of unsaturated fatty acids on the development and proliferation of normal and neoplastic breast epithelium. In: Molecular Interrelations of Nutrition and Cancer, 34th Annual Symposium on Fundamental Cancer Research. Wang, Y., Arnott, M.S. and Van Eys, J. (eds.), M.D. Anderson Hospital and Tumor Institute Monograph (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08229-05 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Dietary Lipids in Increased Incidence of Mammary Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. R. Kidwell	Chief, Cell Cycle Regulation Section	LPP, NCI
Other:	R. A. Knazek	Surgeon	LPP, NCI
	B. K. Vonderhaar	Research Chemist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Branch

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.25	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Epidemiological and experimental studies have implicated dietary fat as a possible etiological agent in the development of human breast cancer. Cumulative evidence suggests that lipids may act directly on the mammary epithelium in fostering neoplasia. The glandular epithelium is buried in a matrix of adipocytes whose contents are modified as a function of dietary fat composition. The interactions between the epithelium and the fat cell have been examined in whole animals and in tissue culture systems. It appears that in response to hormonal stimulation the mammary epithelium recruits fatty acids needed for optimal growth (unsaturated fatty acids) from adjacent fat cells. These fatty acids become enriched in mammary epithelial membrane phospholipids and potentiate the hormonal sensitivity of these cells.

PHS-6040
(Rev. 2-81)

Project Description:

Methods Employed: Ductal and alveolar elements were isolated from rat mammary glands or reduction mammoplasty tissue by means limited collagenase digestion and differential centrifugation. These structures were assessed for growth responsiveness to various fatty acids in primary culture. Effects of proliferative stimuli on the membrane phospholipid composition was determined by fatty acid analysis of membranes prepared from the isolated ducts and alveoli. Fatty acid profiles were determined by gas-liquid chromatography or high pressure liquid chromatography.

Major Findings: 1. Cell membranes prepared from proliferating mammary epithelium are enriched in unsaturated fatty acids. In primary culture, mammary epithelium proliferation is stimulated about two-fold at the optimum concentration of unsaturated fatty acids. At any concentration, saturated fatty acids inhibit cell division. The growth promoting effects of unsaturated fatty acids appear to be manifest via insertion of these fatty acids into membrane phospholipids in place of saturated fatty acid acyl groups. The membranes prepared for ducts and alveoli of animals stimulated with perphenazine to induce mammary epithelial proliferation contained two times as much linoleic acid (mole % of phosphatidyl choline acyl group) as membranes of glands of non-stimualted animals. 2. Prolactin differentially stimulates the uptake of unsaturated fatty acids by mammary epithelium. Isolated ducts and alveoli were incubated in growth medium with a fatty acid complement stimulating the types of fatty acids in the gland in vivo (33% palmitic acid, 14% stearic acid, 25% oleic acid and 28% linoleic acid). After incubation for 24 hrs in the presence of prolactin (100 mg/ml) the cells took up 140 times as much linoleic acid as palmitic acid. A similar difference in magnitude between the uptake of linoleic acid vs stearic acid was observed. Oleic acid was taken up 60 times more efficiently than palmitic acid and 10 times more efficiently than stearic acid when prolactin was added. When prolactin was omitted from the cultures, little fatty acid of any type was utilized from the medium. Thus, hormonal stimulation of the gland results in a differential uptake of the unsaturated fatty acids which stimulate cell growth. 3. Compositional changes occurring in membranes of mammary epithelium in response to a growth stimulus. In addition to changes in the degree of unsaturation of membrane phospholipid acyl groups seen in response to a proliferative stimulus (perphenazine administration) two other indications of proliferation associated membrane changes were observed. Fluorescence polarization measurements of membranes of resting and proliferating epithelium were made. The polarization constant of resting cell membranes was 0.195 ± 0.016 and that of proliferating cell membranes was 0.250 ± 0.007. These differences indicate a considerable increase of membrane microviscosity is associated with growth. The sensitivity of membrane adenyl cyclase to various stimuli was also affected by inducing proliferation. cAMP levels produced/12 min/mg membrane protein were elevated 9 fold over basal levels by GTP + isoproterenol with membranes from proliferating epithelium. With membranes from the epithelium of unstimulated animals, GTP + isoproterenol gave only a 2.5 fold stimulation. Thus compositional changes associated with proliferation increases the sensitivity of epithelial cell membranes to hormonal stimuli.

Significance to Biomedical Research:

In attempts to understand the role of dietary lipids in the development of breast cancer we have begun to analyze the types, quantities and sources of fatty acids taken up by mammary epithelium. Our studies indicate a special role of unsaturated fatty acids in the physiology of the glandular epithelium. These fatty acids are apparently recruited from proximal mammary adipocytes which release their stores of fatty acids in response to a signal from hormonally stimulated epithelium. The fat cells of the gland provide an effective buffer which normally restricts the availability of unsaturated fatty acids to the epithelium. Exceeding the buffering capacity such as may occur in individuals on high fat diets may sensitize mammary epithelium to basal levels of circulating hormones and thereby increase the proliferation rate of the epithelium and expand the population at risk to transformation.

Proposed Course of Research:

A thorough analysis will be made of the compositional changes of mammary epithelial cell membranes from resting and proliferating glands. Attempts will be made to assess which of these changes confer increased hormonal sensitivity to the mammary epithelium.

Publications:

1. Kidwell, W.R.., Knazek, R.A., Vonderhaar, B.K. and Losconczy, I. Effects of unsaturated fatty acids on the development and proliferation of normal and neoplastic breast epithelium. In: Wang, Y., Arnott, M.S. and Van Eys, J. (Eds.), Molecular Interrelations of Nutrition and Cancer, 34th Annual Symposium on Fundamental Cancer Research, M.D. Anderson Hospital and Tumor Institute Monograph, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08230-05 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Hormonal Control of Mammalian Folicular Maturation and Oogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.A. Knazek	Senior Investigator	LPP, NCI
Others:	S.C. Liu	Chemist	LPP, NCI
	A. Rotondi	Visiting Fellow	LPP, NCI
	J.R. Dave	Visiting Fellow	LPP, NCI
	L. A. Liotta	Senior Investigator	LPP, NCI

COOPERATING UNITS (if any)

L. Richardson (IPA) Univ. of Virginia School of Medicine

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cell Cycle Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES X☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

FSH stimulated the synthesis of prostaglandins (PG) E and $F_2\alpha$ by rat ovarian granulosa cells. This dose-related effect was augmented by exogenous hCG. Injections of hCG and/or PRL had no effect without simultaneous FSH treatment. However, infusion of PRL into FSH- or FSH/hCG-treated animals exerted marked effects on PG synthesis. Physiologic replacement doses of PRL resulted in a 2- to 3-fold increase in the rates of PGE and $PGF_2\alpha$ synthesis whereas injections of supraphysiolic amounts of PRL decreased the levels. The effectiveness of PRL over such a narrow concentration range is in agreement with in vitro studies on ovarian steroidogenesis. The role of PG in ovarian function is being studied via a newly devised assay for prostaglandin receptors. The observations suggest that prolactin exerts its effects through the PG cascade and explains, in part, the mechanism by which infertility occurs in hyperprolactinemic women.

PHS-6040
(Rev. 2-81)

Project Description:

Methods Employed: Female Sprague-Dawley rats are hypophysectomized (HIFR) at 21 days of age and implanted subcutaneously with a silastic-encased diethylsilbestrol (DES) pellet. The presence of high concentrations of this estrogenic substance in the absence of gonadotrophins stimulates the proliferation of granulosa cells. Dispersal of such ovaries can be achieved by forcing the tissues through a stainless steel wire mesh and resuspending the granulosal isolates in incubation buffer.

An assay procedure for prostaglandin E and F has been devised consisting of organic solvent extraction, silicic acid column chromatography, and specific radioimmunoassay. Serum progesterone levels were determined by specific radioimmunoassay.

Type IV collagenase activity is measured by solubilization of radiolabeled Type IV collagen in vitro.

Major Findings: Patients suffering from hyperprolactinemia are found to be anovulatory and/or amenorrheic. The reason for this association is unknown. Studies by other investigators have shown that the absence of $PGF_{2\alpha}$ within the ovarian complex prevents release of the ovum in such a manner that would permit subsequent successful fertilization. In addition, Prostaglandin E has been shown to induce neovascularization when implanted in the cornea of the rabbit. Other studies have shown that follicular neovascularization follows the ovulatory surge of LH, a necessary event in forming the well-vascularized corpus luteum, and that women suffering from hyperprolactinemia have an inadequate luteal phase. These observations suggested to us that prolactin might be exerting its effects on the ovary through modification of the PG pathways and prompted the following studies.

Granulosa cells were obtained from hypophysectomized, immature, female rats that had been treated with diethylstilbesterol implants and various s.c. injections of oFSH, PMSG, hCG, oPRL. Cell suspensions were incubated at 37°C for 2 hrs + 10^{-5} M indomethacin. Prostaglandins E and F_2 were extracted from the suspensions, partially purified by chromatography on silicic acid columns and then quantitated by specific RIA. The amounts of PGE and $PGF_{2\alpha}$ synthesized by the DES-treated controls were not significantly altered by treatments with a single injection of 5 I.U. hCG and/or 100 µg oPRL every 4 hours for 2 days prior to sacrifice. Three injections of 100 µg oFSH, however, markedly increased the rates of synthesis of PGE and $PGF_{2\alpha}$. These rates were further increased by 40-100% when a single injection of hCG was added to the FSH treatment. Addition of hourly injections of 50 µg oPRL to either the FSH or FSH ± hGC schedule, however, decreased the synthesis of PGE by 79-80% and $PGF_{2\alpha}$ by 35-56% during the two day treatment period. Doses of oPRL ranging from 0-50 µg were also injected hourly for 48 hours into PMSG + hCG treated animals. This revealed that PG syntheses in the 1.0 µg oPRL group being stimulated to 240-290% that of animals receiving no oPRL. Progesterone levels rose from 0 to 1 ng/ml in the sera of PMSG + hCG-treated animals as the dose of PRL was raised from 0 to 50 µg/hr. These data suggest that the euprolactinemic and hyperprolactinemic states modify ovarian steroidgenesis and the ovulatory process by either accentuating or suppressing the FSH-related stimulation of prostaglandin synthesis.

Other investigators have shown that plasminogen can be activated in the ovary by PGE_1 or PGE_2. This suggested that the anovulatory state of hyperprolactinemia might be caused by alteration of collagenase activity as controlled by prostaglandin levels within the ovary. Preliminary studies show that the ovaries of FSH-treated animals are high in collagenase activity. Treatment with hGC(LH) causes a marked suppression of collagenase Type I and IV within 12 hours. It remains to determine the role of PG in this phenomenon and its relationship to ovulation and corpus luteum formation.

The mechanism by which prostaglandins exert their effects are unknown. However, for these locally active hormones to be effective, they must bind to a receptor site within the ovary. Attempts to study PG binding have resulted in a rapid and highly reproducible PG receptor assay. Radio-labeled prostaglandins E_2 or $F_{2\alpha}$ are incubated with ovarian homogenates in the presence or absence of unlabelled prostaglandins for 1 hr at room temperature. Unbound prostaglandins are removed by adding charcoal to the suspension. After 1/2 hour the charcoal - PG conjugate is removed by centrifuging at ~ 10,000xg atop a 2M sucrose buffer. The charcoal pellet is clipped from the microfuge tube. The remaining liquid phases with the interface-associated membranes are then counted in scintillation counter. The method, rapid and reproducible, has yielded Scatchard curves compatible with either negative cooperativity or the presence of two types of binding sites - high and low affinity.

The above studies indicate that prolactin modifies the synthesis of prostaglandin in the PRL-responsive ovary. This may be the mechanism through which prolactin acts and may also prove to be the physiologic mechanism by which receptor modulation is achieved. Extension of these observations to other PRL-responsive tissues is currently under investigation.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the mechanism by which prolactin acts upon the ovary will enhance our understanding of the role of this mammotrophic hormone in the development of both mammary carcinomata and pathologic states which predispose individuals to its occurrence.

Proposed Course of Research: Granulosa cells subjected to various hormone manipulations will be studied in detail with special attention to alterations in the enzymes and intermediates within the prostaglandin cascade and the modulation of prostaglandin receptor activity.

Publications:

1. Hillier, S.G., Zeleznik, A.J., Knazek, R.A., and Ross, G.T.: Hormonal regulation of preovulatory follicle maturation in the rat. J. Reprod. Fert. 60: 219-229, 1980.

2. Knazek, R.A., Christy, R.J., Watson, K.C., Lim, M.F., Van Gorder, P.L., Dave, J.R., Richardson, L.L., and Liu, S.C.: Prolactin modifies FSH-induced prostaglandin synthesis by the rat granulosa cell. Endocrinology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08233-04 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Morphological Study of the Garfish Olfactory Nerve and Axon-axon Interacters

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. Pinto da Silva Chief, Membrane Biology Section LPP, NCI

COOPERATING UNITS (if any)

Dr. Rodman Miller, Dept. de Morphologie and Institut d'Histologie et d'Embryologie Ecole de Medicine, 1211 Geneve, Switzerland

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.1	0-

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Schwann cell membranes from the myelin sheaths of rat optic and sciatic neverse are observed during differentiation. Freeze-fracture analysis is used to determine the processes of myelin formation. Hexagonal particle rosettes are observed at the periaxonal regions of the Schwann cell plasma membrane in regional correspondence with particle clusters in the axolemma.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To study the mechanism of formation of myelin and the structural basis for possible functional interactions between the cytoplasm of Schwann cells and the axoplasm. To observe the axolemma of non-myelineated axons and search for possible cytoplasmic communication between neighboring axons.

Methods Employed: Rat sciatic and optic nerves are excised, fixed and freeze-fractured. Age of animals 1, 3, 7, 15 days. Garfish olfactory nerves are dissected, fixed and freeze-fractured. Replicas are observed with the electron microscope.

Major Findings: Termination of project pending preparation of manuscript by Dr. R. Miller (Dept. of Histology, Univ. of Geneva, Switzerland).

Significance to Biomedical Research and the Program of the Institute: The investigation of structure and function of membrane is important to understand intercellular communication in normal and transformed cells and many other cellular processes at the level of the plasma membrane and chemistry of surfaces of normal and cancerous cells. Myelination can be a useful model to understand several aspects of demyelinating diseases and secondary nervous lesions in cancer and metabolic disease.

Proposed Course of Research: Manuscript being prepared by Dr. Rodman Miller in Dept. de Morphologie and Institut d'Histologie et d'Embryologie Ecole de Medicine, in Geneva, Switzerland. No new experiments envisaged.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08234-04 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Freeze-fracture Observation of the Normal and Cancerous Prostate Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Pinto da Silva	Chief, Membrane Biology Section	LPP, NCI
Others:	B. Kachar	Visiting Fellow	LPP, NCI
	C. Parkison	Chemist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.0	.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A freeze fracture survey of a prostate gland is made for the first time. Our objectives include: 1) the preparation of a description of cells in the prostate gland, 2) their changes under different conditions of fixation, 3) the observation of membrane events related to secretory processes, 4) the elucidation of temperature and fixation related structural alterations of the membranes of the endoplasmic reticulum and nuclear envelope and 5) the study of rapid changes of the junctional complex of the epithelial cells under controlled experimental conditions.

PHS-6040
(Rev. 2-81)

Project Description:

Methods: Rat ventral prostate gland is dissected, fixed, impregnated in 30% glycerol and freeze-fractured. Platinum-carbon replicas are observed with the electron microscope.

Major Findings: Freeze-fracture morphology of the prostate columnar epithelial cells was investigated. The freeze-fracture technique is utilized to reinvestigate the epithelial organization and the morphology of the different cell zones in the rat ventral prostate. The architecture of the apparatus of synthesis, intracellular transport and secretion is described. Formation of secretory granules appears to be limited to the apical zone and to bypass the Golgi region. Different schedules of fixation demonstrate membrane changes (e.g.: displacement of particles; tight and gap junction proliferation).

Membrane events during apocrine and merocrine secretion of rat ventral prostate epithelial cells were analyzed by freeze-fracture. Early morphological manifestation of both secretory modes involves focal clearance of microvilli characterized by their progressive inclination and attenuation over the apical membrane. Merocrine secretion includes invasion of the apical microfilament web by the secretory vesicle and clearance of membrane particles at the site of its interaction with the apical plasmalemma. During apocrine secretion, a portion of the plasma membrane cleared of microvilli projects into the lumen. Growth of the projection is accompanied by a progressive, but partial, clearing of membrane particles and results in the formation of a large bleb containing dilated endoplasmic reticulum cisternae. Completion of the process involves "degeneration" of the bleb and its release by constriction of a neck, and, possibly, fusion of vesicular or tubular structures. Swelling and blebbing of microvilli are shown to be preparation artifacts.

When carried out at 4°C in contrast to 37°C, glutaraldehyde fixation for routine freeze-fracture specimen preparation results in displacement of membrane particles of epithelial cells of rat ventral prostate. Particle-free areas are observed in endoplasmic reticulum (ER), nuclear envelope, and Golgi membranes; particle aggregation is observed in lateral and basal portions of the plasma membrane. Multilamellar arrangement of lipids can be observed in some of the large particle-free areas. Thin section observation indicates that in the ER, particle-free areas coexist with segregation of cytoplasmic ground substance from the membrane surfaces. Particle-free patches are also observed in the ER membranes of pancreatic tissue fixed at 4°C.

Interactions between the plasma membrane of the different cell types/the basal lamina and/or fiber network are investigated on the extensive face views of membranes. A variety of fixation procedures are used as well as thin section of the tissues. Numerous pits and blebs in the plasma membrane and free vesicles represent material originally organized in the cell structure and released during fixation and specimen preparation.

Incubation at 37°C of excised rat prostate tissue results in massive, proliferative assembly of new tight junctional strands along the entire length of the lateral plasma membrnes of the columnar epithelial cells. The new tight junction elements are assembled within 5 minutes and have an average length 6

times that of those present in the apical tight junction band. Massive assembly occurs in the presence of protein synthesis inhibitors (cycloheximide) or of metabolic uncouplers (dinitrophenol). We conclude that proliferative assembly of tight junction strands involves molecular reorganization from a pool of pre-existing, probably membrane-associated, components. The "fascia occludens", previously described as a genuine cellular structure, as well as other instances involving experimentally induced tight junction proliferation may merely reflect a common response to cellular stress.

Significance to Biomedical Research and the Program of the Institute: Prostate cancer is one of the most prevalent cancers in man. Considerable efforts have been devoted to the study of the structure at the electron microscopic level of normal and malignant prostatic tissues. Most previous studies have used conventional thin sectioning. We attempt for the first time, to study with freeze-fracture, the morphology and dynamics of the membranes of the normal prostate. This step will be followed by a comparative study of prostatic carcinoma in order to evaluate the similarities and differences with their normal counterpart.

Proposed Course of Research: Rapid proliferation of the junctional complex at the lateral membranes of epithelial cells, observed after excision of prostatic tissues, is utilized as an experimental model for studying *in vitro* the disassembly and reassembly of tight and gap junctions. The effect of different variables such as temperature, ionic strength, pH, Ca^{++} and substances utilized during specimen preparation (fixatives and crioprotectants) are evaluated in freeze-fracture replicas.

Publications:

1. Kachar, B., Serrano, J.A. and Pinto da Silva, P. Particle displacement in epithelial cell membranes of rat prostate and pancreas induced by routine low temperature fixation. Cell Biol. Int. Reports. In press.

2. Kachar, B. and Pinto da Silva, P. Freeze-fracture study of rat ventral prostate secretory mechanisms in the epithelial cell. Anatomical Record. 198:: 549-565, 1980.

3. Kachar, B. and Pinto da Silva, P. Freeze-fracture study of rat ventral prostate: The columnar epithelial cell. Am. J. Anat. (in press).

4. Kachar, B. and Pinto da Silva, P. Rapid, massive assembly of tight junction strands. Science (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08235-04 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Freeze-fracture Observation of Platelet Membranes during Clot Formation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. Pinto da Silva Chief, Membrane Biology Section LPP, NCI

COOPERATING UNITS (if any)

J.F. David-Ferreira, M.H. Miranda, Laboratory of Cell Biology, Gulbenkian Institute of Science, Oeiras

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Application of fracture-label technques to the study of the partition and distribution of lectin binding sites on the faces freeze-fractured human platelet membranes from normal and diseased donors.

PHS-6040
(Rev. 2-81)

Project Description:

Methods: Human platelets are isolated, fixed, embedded into a cross-linked matrix of BSA, impregnated in glycerol, frozen and freeze-fractured. After thawing the fractured cells are treated with wheat germ agglutinin, concanavalin A and lens culinaris agglutinin. Specimens are labelled with colloidal gold and processed for thin section or critical pount drying and platinum relicated.

Major Findings: The early stages of platelet adhesion and aggregation are surface-mediated phenomena and involve three major glycoprotein families. In a preliminary set of experiments, we demonstrated that among these glycoproteins, Gl_1 which is linked to the adhesion process to the subendothelim and which binds specifically the wheat germ agglutin lectin (WGA) is almost completely partitioned with the external leaflet of the membrane. On the contrary, Gl_3 involved in the aggregation process seems to be an intrinsic protein with a major part located inside the external leaflet and a minor part inside the inner leaflet of the membrane, as suggested by the concanavalin A colloidal gold complex distribution.

Significance to Biomedical Research and the Program of the Institute: Despite the fact that membrane glycoprotein composition of the human blood platelet is now fairly well known from a biochemical point of view, only a few data on their spatial an arrangement inside the membrane are available. The ability of the fracture label technique to visualize both the inner core of the membrane and the cytochemical markers should be useful in this attempt to define the spatial arrangement of the glycoproteins. This approach could shed new lights on the data obtained by 80S-PAGE, enzymatic digestion and iodination techniques.

Proposed Course of Research: To complete our observations using fracture-label techniques. To compare with the labelling characteristics of platelets derived from patients missing important glycoproteins from their plasma membranes (Trombastenia; Bernard-Soulier).

Publications:

1. Miranda, M.H., David-Ferreira, J.F., Pinto da Silva, P. and Temos, J.C. Freeze-fracture study of the human blood platelets. Cien. Biol. (Portugal) 5: 7a, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08240-03 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Growth, Metastasis and Regression of Human Breast Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sam Shafie	Visiting Associate	LPP, NCI
Others:	Luis Franco	Guest Worker	LPP, NCI
	Mary McManaway	Bio. Lab. Tech.	LPP, NCI
	Spring Randolph	Bio. Lab. Tech.	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathophysiology

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Office of the Chief

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.00	2.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Solid mammary tumors were formed in nude mice by s.c. injections of a number of human breast cancer cell lines. Tumor formation was estrogen-dependent. After the tumor is established estrogen withdrawal failed to induce tumor regression. Host modification and aromatase inhibitor studies indicated that the lack of tumor regression was not due to tumor paraendocrine activity nor to a persisting host modification following estrogen withdrawal. Tumor regression was obtained by adriamycin treatment or treatment with the antiestrogen, tamoxifen, which competes for estrogen receptor. Tumors were ten times more sensitive to tamoxifen or adriamycin when the tumor host was deprived of estrogen. This was demonstrable in all tumor lines examined and establishes a role for estrogen in influencing the sensitivity of breast carcinoma to adriamycin. Adriamycin was consistently effective in competing with estrogen for estrogen receptor. The specificity of this competition is now being examined.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: 1) To identify the hormonal requirements for the induction of human solid breast tumors in nude mice and the progression of the disease (metastasis); 2) to identify the mechanism of hormone action by extrapolating between in vitro and in vivo (nude mice) environments whereby the host related determinants that could influence tumor growth are also considered, and 3) to utilize the results obtained from the above as a basis for endocrine additive and ablative therapy and to combine endocrine and chemotherapies for the induction of tumor regression.

Methods Employed: The human breast cancer lines MCF-7 and ZR-75 were used for the induction of tumors in athymic nude mice. Dependence on or stimulation by hormones was tested by either hormone withdrawal (surgical removal of endocrine tissues) or hormone administration through injection or by placing a hormone pellet in the suprascapular region of the host mouse. Metastases was monitored by histology and invasiveness of cultured cells into agar was tested by a method developed by L. Liotta.

Hormonal and drug effects were also studied in the continuous culture and in primary cultures derived from the enzymatic (collagenase: hyaluronidase) dissociation of MCF-7 tumors.

Major Findings:

1. The MCF-7 human breast cancer cell line has been shown to produce solid tumors with a 100% take in intact athymic nude mice. Tumors failed to develop in ovariectomized (OVX) mice or in intact mice treated with the aromatase inhibitor (A.I.) 4-hydroxyandrostenedione. At concentrations of 10^{-9} to 10^{-8}M, A.I. had no effect on MCF-7 growth in tissue culture. MCF-7 tumors developed and grew 5 to 6-fold faster in intact or OVX mice supplemented with 17β-estradiol (E_2) in the form of a pellet (100 µg).

Withdrawal of the E_2 pellet from OVX mice bearing actively growing 5 week-old tumors (0.8-0.9 cm^3) resulted in a temporary growth arrest that lasted about 10 days. Tumors then started to regrow at a rate comparable to that seen in mice that retained their E_2 pellets. The combined effect of E_2 withdrawal and A.I. treatment also failed to produce tumor regression. MCF-7 tumors showed close similarity in histopathology and estrogen receptor level to the continuous tumor line. The possibility that absence of regression following E_2 withdrawal may only be due to a lasting modification of the host environment by prior E_2 treatment was ruled out by the following experiments: 1) When the E_2 pellet was removed from tumor bearing mice and a new MCF-7 inoculum was injected on opposite side of the established tumor, new tumors developed, and 3) When the E_2 pellet was kept and a new inoculum injected in the presence or absence (excised) of established tumor, new tumors also developed. The results indicate that MCF-7 cells, after having been established in the nude mouse in an E_2-dependent process, manage to grow independent of host or exogenous estrogen.

2. The MCF-7 human breast cancer cell line is growth-unresponsive to estrogen in culture. Paradoxically, growth and solid tumor formation by these cells in nude mice are dependent on estrogen. Tumors fail to develop in ovariectomized mice, but do develop in intact mice, and in ovariectomized mice given estrogenm. Primary

cultures derived from MCF-7 tumors revert to estrogen unresponsiveness. However, tumor formation by these cultures retransplanted in nudes requires estrogen. The estrogen binding capacity and affinity of the continuous culture, the solid tumor and the primary cultures therefrom, are similar. This suggests that mammary growth in vivo is subject to inhibition that can be overcome by estrogen.

3. As an attempt to account for the in vivo dependence on estrogen for growth of MCF-7, the effects of estrogen treatment on levels of natural killer (NK) cell activity were examined. Two to four weeks following estradiol treatment of 4- to 6-week-old mice, NK cell activity was strongly depressed. In contrast, NK activity in the spleens of ovariectomized mice, which had undetectable levels of serum estrogen, was quite high. NK activity in untreated, intact mice was intermediate. Serum estrogen levels were 4×10^{-10} M in intact mice and 2×10^{-9} M in mice treated with estradiol. These results indicate that estradiol downregulates NK activity in nude mice and suggest that the inability of MCF-7 cells to grow in vivo without estradiol may be due, at least in part, to the involvement of NK activity in the host antitumor defense mechanism.

4. The sensitivity to adriamycin (ADR) cytotoxicity of 5 human breast carcinoma cell lines was tested and compared with their estrogen receptor (ER) status and growth rate. The MCF-7, ZR-75-1 and $T_{47}D$ cell lines are classified as ER positive with a doubling time of 33 hr, 34 hr, and 32 hr, respectively, in minimal essential medium (MEM) + 10% fetal bovine serum (FBS). The MDA-MB-231, and ZR-75-31A cell lines are ER negative with a doubling time of 16 hr, and 14 hr, respectively, in the same media. Sensitivity to ADR was examined by plating 1×10^6 cells/T_{25} flask in MEM with 10% FBS and allowed to attach for 48 hr. Next, the cells were exposed for 48 hr to MEM+10% FBS$\pm$ 1.5, 2.5, 3.0, or 4.0 µg/ml ADR before they were switched back to MEM+10% FBS for additional 48 hr. The effect of ADR on growth was based on cell count and DNA mass with the following results using 1.5 µg/ml ADR. (mean$\pm$s.e.; N=6): 1) MCF-7, control=100$\pm$7.47% (3.12×10^6 + 2.33×10^5 cells); control + ADR, 47.63$\pm$ 3.00%, 2) ZR-75-1, control=100$\pm$2.80% ($4.74 \times 10^6 \pm 1.33 \times 10^5$ cells); control + ADR 46.36$\pm$ 3.43%, 3) $T_{47}D$, control = 100$\pm$ 1.33 $\times 10^5$ cells); control + ADR = 44.44 $\pm$ 5.56%, 4) MDA-MB-231, control = 100$\pm$9.83% ($2.97 \times 10^6 \pm 2.92 \times 10^5$ cells); control + ADR = 8.60$\pm$1.01%, 5) ZR-75-31A, control = 100$\pm$3.00% ($7.46 \times 10^6 \pm 2.25 \times 10^5$ cells); control + ADR = 14.36 $\pm$ 1.27%. Increasing concentrations of ADR produced higher levels of growth inhibition in all cell lines.

The results suggest that 1) breast carcinoma cells that are ER negative are more sensitive to ADR than breast carcinoma cells that are ER positive and 2) the growth rate appears to be a determining factor in the tumor cell sensitivity to the drug.

5. The tumor host estrogen status was shown to influence the sensitivity of tumor cells to drug treatment. Adriamycin produced human breast tumor regression in nude mice but adriamycin cytotoxicity was enhanced several fold upon the simultaneous estrogen withdrawal at the time of treatment.

6. Plasminogen activator activity was shown to be modified by endocrine manipulations in hormone-dependent DMBA-induced mammary tumors. The enzyme activity decreased several fold in tumors excised from rats following ovariectomy or the induction of diabetes.

Significance to Biomedical Research and the Program of the Institute:
The hormonal environments required by human breast cancer cells MCF-7 to produce solid tumors and induce their regression in nude mice have been outlined. The results suggest that host-tumor interactions as well as hormone responsiveness of human breast cancer can be studied in the nude mouse system. The studies also revealed that mammary growth in vivo may be subject to inhibition that can be overcome by estrogen thus introducing a new perspective for studying the mechanism of sex steroid action in breast cancer.

Estrogen and insulin have been shown to be essential requirements for tumor growth and metastases and in the regulation of plasminogen activator activity. Attention is called to the co-existence of enhancement in collagenase production and metastasis formation controlled by estrogen and insulin.

The studies with insulin-methotrexate therapy in vitro and with estrogen-adriamycin in vivo suggested that tumor cell sensitivity to chemotherapy could be selectively increased by manipulating the activity of tumor cell metabolic pathways that determine drug cytotoxicity.

Proposed Course of Research:

Project will be terminated with this report.

Publications:

1. Shafie, S.M. Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science 209: 701-702, 1980.

2. Shafie, S.M. and Liotta, L.A. Formation of metastasis by human breast carcinoma cells (MCF-7) in nude mice. Cancer Let. 11: 81-87, 1980.

3. Shafie, S.M. and Hilf, R. Insulin receptor levaels and magnitude of insulin-induce responses in DMBA-induced mammary tumors in rats. Cancer Res. 41: 826-829, 1981.

4. Alabaster, O., Vonderhaar, B.K., and Shafie, S.M. Metabolic modification by insulin enhances methotrexate cytotoxicity in MCF-7 human breast cancer cells. Eur. J. Cancer, in press.

5. Shafie, S.M. and Grantham, F.H. Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J. Natl. Cancer Inst., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08247-03 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Basement Membrane Collagen Degradation and Its Role in Basement Membrane Physiology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. Liotta	Expert	LPP, NCI
Others:	S. Garbisa	Visiting Scientist	LPP, NCI
	C. Foltz	Research Chemist	LC, NIAMDD
	R. Brundage	Biologist	LPP, NCI

COOPERATING UNITS (if any)

Laboratory of Developmental Biology and Anomalies, NIDR and Laboratory of Chemistry, NIAMDD

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

There are at least five genetically distinct collagen types whose degradation may be controlled independently. The initial step of collagen degradation is performed by collagenase. We were the first to find that type IV basement membrane collagen and type V collagen is not degraded by human skin collagenase suggesting that a separate collagenase may degrade types IV and V collagen. A collagenase which preferentially degrades type IV collagen has been derived from metastatic tumor cells and from mammary epithelium. This collagenase has been purified 1000-fold and its cleavage products have been partially characterized. We are further studying the secretion rate of this enzyme by a wide variety of cell types both normal and malignant.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The objectives of this project are to purify and characterize type IV collagen degrading enzymes and to study their biological significance in tumor invasion and metastases, angiogenesis, embryology and diabetes.

Methods Employed: Crude collagenase is obtained from a) serum-free cultures of highly metastatic mouse tumor, b) serum free culture of minced involuting mammary gland, c) serum-free cultures of cultured human breast carcinoma and d) cultures of rat mammary ducts and alveoli. The enzyme activity is precipitated with ammonium sulfate and purified by concanavillin A sepharose molecular sieve and collagen affinity chromatography. Collagenase activity is studied on labelled and unlabelled purified collagens I, II, III, IV and V. Degradation products are studied by gel electrophoresis. Enzyme specificity is studied by injecting the purified enzyme with or without inhibitors into various anatomical sites in the mouse.

Major Findings: A neutral protease has been extracted from the media of cultured metastatic tumor cells and purified approximately 1000 times after sequential ammonium sulfate fractionation, concanavalin A column chromatography, and molecular sieve chromatography. The protease has an apparent molecular weight of 70-80,000, is inactive at acid pH, requires trypsin activation, and is inhibited by ethylenediamine tetraacetic acid, but not phenylmethyl-sulfonyl fluoride, n-ethyl maleimide, or soybean trypsin inhibitor. The enzyme produces specific cleavage products for both chains of type IV collagen isolated without pepsinization and apparently cleaves at one point in a major pepsin extracted chain of placenta type IV collagen. The partially purified enzyme fails to significantly degrade other collagens or fibronectin under digesting conditions in which specific reaction products are produced for type IV collagen. The reaction products indicate a single cleavage in the body of the type IV collagen molecule.

A wide variety of human and rodent cells were studied for type IV collagen degrading activity. Inflammatory cells endothelial cells, epithelial cells but not normal connective tissue cells exhibited enzyme activity. Metastatic tumor cells secreted enzyme activity in proportion to their ability to produce spontaneous metastases in vivo. Antibodies to type IV collagenase consistently inhibited metastases from i.v. injected tumor cells.

Latent collagenase was present in tumor interstitial fluid. collagenase activity was enhanced in MCF-7 breast carcinoma cultured with insulin.

Significance to Biomedical Research and the Program of the Institute: Breakdown of basement membranes occurs during the transition from in situ to invasive carcinoma and during penetration of vessel walls by metastasizing tumor cells. The basement membrane collagen degrading enzyme identified and purified in this project may play a role in the cancer invasion process. Detection of the enzyme may be a means of predicting the metastatic potential of a tumor. Furthermore, human breast carcinoma cells have been shown to secrete latent collagenase which degrades stroma type I collagen. Hence the specificity of different collagenase species elaborated by tumor cells may influence their pattern of invasion.

Proposed Course of Research: a) Purification of type IV collagen degrading enzyme to near homogeneity using HPLC and isoelectric focusing b) purifying antibodies against this enzyme. c) Use of the enzyme to elucidate the structure of type IV collagen. d) The effect of type IV protease on the morphologic structure of the basement will be studied by electron microscopy.

Publications:

1. Liotta, L.A., Garbisa, S., Tryggvason, K. and Wicha, M.: Correlation of metastatic behavior with tumor cell degradation of basement membrane collagen. In Tumor Progression. Ray Crispen (ed.), Chicago, (1980).

2. Liotta, L.A., Garbisa, S., Tryggavason, K., Robey, P.G. and Murray, J.C.: Interaction of metastatic tumor cells with basement membrane colalgen. In Metastatic Tumor Growth. E. Grundmmann (ed.) Pub. G. Fischer, Stuttgart-New York, vol. 4: 21, 1980.

3. Garbisa, S. Kniska, K., Tryggavason, K., Foltz, C. and Liotta, L.: Quantitation of basement membrane collagen degradation by living tumor cells in vitro. Cancer Letters 9: 359-366, 1980.

4. Gullino, P.M. and Liotta, L.A.: Cell shedding by tumors. In Bone Metastasis. L. Weiss and H.A. Gilbert (eds), Pub. G.K. Hall, Boston, 1980.

5. Liotta, L.A., Tryggvason, K. Garbisa,d S., Gehron-Robey, P., Abe, S.: Partial purification and characterization of a neutral protease which cleaves type IV collagen. Biochemistry 20: 100-108, 1981.

6. Garbisa, S., Tryggvason, K., Foidart, J.M., Liotta, L.A.: Assay for radiolabeled type IV collagen in the presence of other proteins using a specific collagenase. Anal. Biochem. 107: 187-192, 1980.

7. Shafie, S. and Liotta, L.A.: Metastases of MCF-7 Human Breast Carcinoma in Nude Mice. Cancer Letters 11: 81-87, 1980.

8. Salomon, D., Liotta, L.A., Kidwell, W.R. Differential response to growth factor by rat mammary epithelium plated on different collagen substrata in serum-free medium. Proc. Natl. Acad. Sci. 78, pp. 382-386, 1981.

9. Lewko, W., Liotta, L.A., Wicha, M.S. Vonderhaar, B. and Kidwell, W.R. Sensitivity of NMU induced rat mammary tumors to cis-hydroxyproline, an inhibitor of collagen production. Cancer Research, 1981 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08249-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Hormonal Control of Growth and Differentiation of Normal and Neoplastic Mammary Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W. R. Kidwell,	Chief, Cell Cycle Regulation Section	LPP, NCI
Others: W. M. Lewko	Instructor, Dept. Biochem. Univ. of Lousiville (Formerly LPP, NCI)	
L.A. Liotta	Surgeon	LPP, NCI
J. Zweibel	Research Associate	LPP, NCI
D. Salomon	Expert	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cell Cycle Regulation Section

INSTITUTE AND LOCATION

NCI, NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hormonal dependency of normal mammary epithelium is a retained characteristic of some but not most mammary tumors. The mechanism by which loss of hormone dependency occurs is unknown but is of great clinical significance for the breast cancer patient. In attempts to elucidate this mechanism, comparisons are being made of the 1) cellular composition, 2) the extracellular matrix production, 3) the growth factor production and requirements and 4) the characteristics of the receptor systems of hormone dependent (primary NMU-induced) rat mammary tumors. The primary tumors consist of both epithelial and myoepithelial-like cells, are estrogen receptor positive, synthesize and require type IV collagen for growth and produce a sarcoma growth factor-like substance substance. The hormone independent, transplantable NMU tumor possesses no myoepithelial component, neither synthesizes nor requires type IV collagen for growth, is estrogen receptor negative and produces little or no sarcoma growth factor-like substance. We tentatively propose that hormone dependency may be related to the presence of the myoepithelial-like cells in tumors. These cells synthesize type IV collagen and the sarcoma growth factor-like component in culture.

Project Description:

Methods Employed: Steroid hormone receptors were measured by the hydroxylapatate method of McGuire. Collagen synthesis requirements for growth were determined in vivo and in vitro by using of the specific collagen formation inhibitor, cis-hydroxyproline. Collagen synthesis and the determination of collagen type synthesized was assessed by gel electrophoresis following labeling the isolated tumor cells with ^{3}H-lysine for 6 hrs. Alternatively, the type of collagen synthesized was determined by indirect immunofluorescence with specific antibodies.

Major Findings: 1. Loss of estrogen receptor responsiveness by isolated tumor epithelium. During isolation the estrogen receptors of primary NMU tumor cells were found to be unstable. Incubation of the tumor at 37°C caused an 80% reduction of receptors but 80% retention was achieved by performing tumor cell dispersion with collagenase at 30°C. Not only were receptor levels lost by dispersion at 37°C but the estrogen responsiveness as well. Cells isolated at 37°C lost their sensitivity to the antiestrogens, tamofixen and nafoxide compared to cells isolated at 30°C. Nafoxidine (400 nM) inhibited the growth of cells isolated at 30°C by 70%. The same concentration of the inhibitor reduced the growth rate of cells isolated at 37°C by only 6%. The results with tamoxifen (400 nM) were very similar. The mechanism of the loss of estrogen receptors during cell isolation is unknown. The loss is largely irreversible since the receptor levels are not regained in culture. The results show that estrogen dependency of mammary cell cultures can be readily demonstrated if the cells are isolated at the proper temperature. 2. Collagen synthesis requirement for tumor cell growth. Both normal mammary epithelium and the epithelium from primary mammary tumors induced by DMBA or NMU synthesize type IV collagen. The growth of the epithelium from either source (in vivo or in vitro) is arrested by cis-hydroxyproline, a compound which blocks collagen deposition. In contrast, the transplantable NMU or DMBA tumors neither synthesize type IV collagen nor show growth inhibition by the proline analogue (in vivo studies). Other transplantable rat mammary tumors are sensitive to cis-hydroxyproline. A 30-50% inhibition of growth rates are seen with MTW9, R3230 AC and 13762 tumors. The sensitivity of the tumors to cis-hydroxyproline appears to be related to the ability of the tumors to synthesize a basement membrane. All of the sensitive tumors make type IV collagen while the insensitive tumors do not. 3. Sarcoma growth factor-like substance produced. Primary tumors induced by NMU or DMBA produce high amounts of a factor(s) that competes for EGF receptor binding on cells, confers anchorage independent growth properties to NRK cells and is a potent mitogen for normal rat mammary epithelium, 3T3 and NRK-F49 cells, and chick embryo fibroblasts. The factor is heat labile, pepsin sensitive and has an apparent molecular weight of 70,000. Transplantable NMU and DMBA tumor extracts have little or none of the factor compared to the primary tumors. The primary tumor cells have been fractionated into 2 cell types and the myoepithelial cell population shown to produce the factor. Separation of the latter cells from tumor epithelium is achievable by plating the mixed cell population in Ca^{+2} free medium. Growth of the myoepithelial cells or epithelial cells alone is poor but dramatically improved where the two cell types are recombined, indicating cell interaction is requisite for optimum proliferation.

Significance to Biomedical Research and the Program of the Institute: These studies will help to identify which hormones are mitogenic for normal and neoplastic mammary tissues and provide valuable insight into the roles of individual cell types in the growth process and into the development of hormone independency of mammary tumors. Such information may have significant implications for the treatment of human breast cancer.

Proposed Course of Research: Evidence to date is suggestive of growth control mediated by extracellular matrix production by the glandular myoepithelial compartment. More direct evidence for this will be sought by separating and combining the epithelial and myoepithelial cell populations to determine how they interact in a proliferative mode. Additionally, attempts will be made to selectively destroy the myoepithelial cells in mixed cell cultures followed by analysis of the effects on epithelial cell proliferation by autoradiographic techniques. Finally, the hypotheses formulated from studies of rodent mammary cells will be extended to that of humans using reduction mammoplasty tissue. Further effort will be directed at identifying and characterizing the growth factors made by the primary tumor and the importance of the factor in hormone dependency. Attempts will be made to modulate production of the factor by supplementing the growth medium of primary cultures with progestins, estrogens, corticosteroids and pituitary hormones.

Publications:

1. Kidwell, W. R., Wicha, M. S., Liotta, L. A. and Salomon, D. Hormonal controls of collagen substratum formation by cultured mammary cells: Implications for growth and differentiation. In Shields, R., Jiminez de Asua, L. and Levi-Montalcini, L. (Eds.): Control Mechanisms in Animal Cells. Raven Press. pp. 333-340, 1980.

2. Kidwell, W. R., Wicha, M. S., Salomon, D. and Liotta, L. A. Differential recognition of basement membrane collagen by normal and neoplastic mammary cells. In: McGrath, C. M., Brennan, M. J. and Rich, M. A. (Eds.): Cell Biology of Breast Cancer, Academic Press, New York. pp. 17-32, 1980.

3. Wicha, M. S., Liotta, L. A., Vonderhaar, B. K. and Kidwell, W. R. Effects of inhibition of basement membrane collagen deposition on rat mammry gland development. Develop. Biol. 80, 253-266, 1980.

4. Wicha, M. S., Liotta, L. A., Lewko, W. M. and Kidwell, W. R. Blocking basement membrane collagen deposition inhibits the growth of 7,12-DMBA induced rat mammary tumors. Cancer Letters 12: 9-21, 1981.

5. Salomon, D. S., Liotta, L. A. and Kidwell, W. R. Differential growth factor responsiveness of rat mammary epithelium plated on different collagen substrata. Proc. Nat. Acad. Sci., USA 78: 382-386, 1981.

6. Lewko, W. M., Liotta, L. A. Wicha, M. S., Vonderhaar, B. K. and Kidwell, W.R. Sensitivity of N-nitrosomethylurea-induced rat mammray tumors to cis-hydroxyproline. Cancer Research. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08250-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis and Development of Freeze-Fracture Methods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Pinto da Silva	Chief, Membrane Biology Section	LPP, NCI
Others	B. Kachar	Visiting Fellow	LPP, NCI
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	J. Chevalier	" "	LPP, NCI
	M.L. Nogueia	" "	LPP, NCI
	C. Parkison	Chemist	LPP, NCI
	C. Brown	Chemist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Biology Section

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NCI, NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	1.5

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

New "fracture-label" techniques have been developed that permit for the first time, direct label of the fracture faces of plasma and intracellular membranes as well as cross-fractured cytoplasm. The epistemological basis of morphology, in particular that of freeze-fractured and freeze-etched preparations is investigated.

PHS-6040
(Rev. 2-81)

Project Description:

Methods: Cells are embedded in bovine serum albumen and then crosslinked into a gel by addition of glutaraldehyde. The gels are impregnated in glycerol, rapidly frozen in liquid nitrogen cooled Freon 22 and fractured under liquid nitrogen. The specimens are then tawed and labelled with a variety of lectins (in the form of ferritin or coloidal gold conjugates) and processed for thin section ("thin section fracture-label") or critically point dried and observed as platinum carbon replicas ("critical point dried fracture label").

Major Findings: We showed that the newly, developed method of quick-freezing by a slamming device can induce worked alterations in the architecture of plasma membranes and secretory besides and do not faithfully represent intermediate changes associated with the fusion events during secretion. We concluded that: 1) The intrinsic advantages in the application of rapid freezing methods to study rapid membrane changes associated with secretory events are restricted to systems where an instant stimulus induces a massive, synchronous, response. 2) Successful observation and detection of intermediate stages associated with membrane fusion depends on the signal to noise ratio of the visual images. In consequence, it may be necessary to intervene in the secretory process creating conditions which may lead to increased frequency and conspicuousness of membrane fusion intermediates. 3) During quick freezing by a slamming device, specimen impact precedes freezing and can cause disruption of critical aspects of membrane architecture including those related to fusion of secretory vesicles with the plasma membrane. 4) The current understanding of the sequence of events during secretion associated membrane fusion is, at present, derived from chemically fixed specimens. Quick freezing by a slamming device has been unable to provide additional insights into this process and, in fact, can be interpreted to confirm previous data obtained from chemically fixed tissues and cells.

New "fracture-label" methods are discovered which permit the study of the distribution and partition of lectin binding sites, membrane antigens and receptors anionic sites on the fracture faces of biological membranes. Initial application of these methods to the study of erythrocyte membranes show that both fracture faces are rich in anionic sites, including sites newly revealed by the fracture process. In addition, Band 3 component, the principal transmembrane protein in erythrocyte membranes (and its anion carrier) partitions preferentially with the protoplasmic A face and inconsequence is dragged, during fracture, across the outer half of the membrane. The technique was also applied to a variety of other cells and tissues showing, in addition that the exoplasmic (cisternal) faces of nuclear envelope and endoplasmic reticulum membranes are rich in cancanavalin A binding sites. The two methods developed, "thin section fracture-label" and "critical point dried fracture label" provide complementary views of the distribution of the label.

Significance to Biomedical Research and the Program of the Institute: Fracture label techniques, developed in this project, give, for the first time, generalized access to the labelling of freeze fractured preparations. Technically, the methods are accesible and, we expect, will be of widespread use. For instance, they will permit the comparison of lectin labelling partitions and distribution in normal and cancerous cells. It is thought that differences in expression of glycoproteins observed on the outer surfaces of transformed cells have their origin

with the cell at the level of the endoplasmic reticulin and gogi apparatus where synthesis and glycosilation occurs. With this technique we will be able to determine the existence of membrane traversing proteins in other cells (we are currently investigated lymphoid cells) where they may be associated to important antigens (e.g. transplantation antigens).

Proposed Course of Research: Application and development of fracture label techniques to the plasma and intracellular membranes of other cells and tissues.

Publications:

1. Pinto da Silva, P. and Kachar, B. Quick freezing vs. chemical fixation: capture and identification of membrane fusion intermediates. Cell Biology Int. Reports. 4: 625-640, 1980.

2. Pinto da Silva, P. and Kachar, B. Disruption of critical aspects of membrane architecture during quick freezing by a slamming device. J. Cell Biol. (in press).

3. Pinto da Silva, P., Parkison, C. and Dwyer, N. Fracture-label: cytochemistry of freez-fracture faces in the erythrocyte membarane. Proc. Nat. Acad. Sci. 78: 343-347, 1981.

4. Pinto da Silva, P., Parkison, and Dwyer, N. Freeze-fracture: Thin section of cells and tissues after labelling of fracture faces. J. Histochem. Cytochem (in press).

5. Pinto da Silva, P., Kachar, B., Torrisi, M. Rosaria, Brown, C. and Parkison, C. Freeze-fracture cytochemistry: replicas of critical point dried cells and tissues after "fracture-label." Science (Wash.) (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08251-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Growth Factor Production by Neoplastic Rat Mammary Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Katari	Staff Fellow	LPP, NCI
	J. Zweibel	PHS Fellow	LPP, NCI
Others:	D. S. Salomon	Expert	LPP, NCI
	W. R. Kidwell	Chief, Cell Cycle Regulation	LPP, NCI
	P. M. Gullino	Chief	LPP, NCI

COOPERATING UNITS (if any)

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Laboratory of Pathophysiology

SECTION

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.9	0.9

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER X

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The program's objectives are to: 1) isolate and characterize a mammary tumor factor(s) (MTF) which resemble epidermal growth factor (EGF) and sarcoma growth factor (SGF) from chemically-induced rat mammary tumors and from the conditioned medium of primary cultures of mammary tumor epithelial cells; 2) to determine and compare the biological, biochemical and antigenic properties of MTF to EGF and SGF; 3) to compare the effects of MTF on the growth and differentiation of normal rat mammary epithelium (RME), mouse embryonal carcinoma (EC) cells, and chick, mouse and rat fibroblasts and 4) to delineate the relationship of MTF production in preneoplastic cells and primary and transplantable hormone-dependent and and independent tumors to tumor promotion and growth.

PHS-6040
(Rev. 2-81)

Project Description:

Transformation of a variety of cells with RNA tumor viruses results in the failure of these cells to bind or respond to exogenous growth factors such as EGF or MSA because of the production of endogenous growth promoters such as sarcoma growth factor (SGF), which autostimulate these cells to continuously grow. If ectopic production of these or related growth factors is a general property of preneoplastic or neoplastic cells, then such factor(s) should be present in primary or transplantable, chemically-induced mammary tumors as well as in the conditioned medium of primary cultures of these tumor cells in vitro.

Objectives: These studies are designed to isolate and characterize ectopic growth factors from rat mammary tumors and from the conditioned medium of tumor cells in vitro and to ascertain if ectopic growth factor production is unique to mammary tumor cells or if normal RME cells also produce a similar or different set(s) of factors.

Methods Employed: A. Collection and Fractionization: Mammary tumors, mammary glands from perphenazine-treated rats, and other tissues will be extracted by an acid-ethanol procedure described by Roberts et al., PNAS, 77, 3495, 1980, for the isolation of transforming growth factors (TGF) from human carcinomas. The acid soluble material will be fractionated through a series of Amicon ultrafilters following dialysis against 1% acetic acid and further separation achieved by gel filtration, ion-exchange chromatography and preparative isoelectric focusing. Pooled material will be tested in several bioassay, radioreceptor and radioimmunoassay systems. Conditioned medium from primary cultures of tumor cells grown in serum-free medium will also be fractionated and tested in a similar manner. B. Bioassays: a) Ability to stimulate anchorage-independent growth (colony formation) of normal rat kidney (NRK) cells in soft agar; b) ability to enhance the proliferation of chick embryonic fibroblasts (CEF), mouse 3T3 fibroblasts, NRK and RME cells; c) ability to modulate EC differentiation with this parameter being monitored by the synthesis and turnover of type IV collagen and laminin. C. Biochemical assays: a) ability to compete with [^{125}I]EGF, [^{125}I]MSA or [^{125}I]insulin for specific binding to EGF, sommatomedin or insulin receptors on EC cells or A431 human epidermoid carcinoma cells; b) ability to cross-react with either EGF or MSA in radioimmunoassays (RIA) using antisera to EGF, MSA or insulin and c) comparison of gel filtration, ion-exchange and electrophoretic properties to known growth factors.

Major Findings: The serum-free conditioned medium from primary cultures of 7,12-dimethylbenzanthracene(DMBA)-induced rat mammary adenocarcinoma cells contains a heat labile, acid-stable, protein factor(s) (mw approx. 80,000 daltons) which is able to compete with [^{125}I]EGF but not [^{125}I] insulin for radioreceptor binding on mouse EC cells. The factor(s) is antigenically distinct from EGF and induces the growth of NRK cells in soft agar as colonies. It is a mitogen for NRK and RME cells in monolayer culture, reduces the serum requirement for these cells in vitro and promotes the loss of contact inhibition of NRK cells. A similar activity (or set of activities) can be isolated directly from primary DMBA or nitrosomethylurea (NMU)-induced mammary tumors. Using several primary and transplantable mammary tumors, the factor(s) is highest in primary or transplantable tumors which are hormone-dependent. Activity is substantially lower in transplantable hormone-independent tumors tested (DMBA, NMU or MTW9A). No

activity can be detected in proliferating mammary glands from perphenazine treated rats but human breast epithelium from reduction mammoplasty produces the factor(s) in primary culture. The epithelium from primary DMBA induced tumors has been partially fractionated into its two major cell components, epithelial cells and basal (or Myoepithelial) cells by selective attachment to culture dishes in the absence of calcium. The basal cell cultures (90-95% pure) and the epithelial cell fraction (which is still contaminated with appreciable basal cells) both produce the factor(s). The factor(s) is apparently a protein. It is destroyed by pepsin

Significance to Biomedical Research and the Program of the Institute: Transformation of cells in vitro by chemical or viral agents has been generally assumed to correlate with the acquistion of tumorigenicity in vivo. A variety of parameters are known to be associated with the transformed phenotype such as a loss in contact inhibition of growth; an increase in DNA synthesis, cellular proliferation and saturation density; ability to grow in soft agar and failure of cells which are able to differentiate to do so upon transformation. Several of these properties can be reversibly produced in nontransformed cells by factors which are synthesized by neoplastic cells in vitro. The ability to mimic the transformed phenotype by administration of these epigenetic, hormonal-like "transformation peptides" implies that they may be involved in the early stages of conversion of a normal cell to a preneoplastic cell. Autonomous production of such factor(s) may be involved in the acquisition of unrestrained growth by cancer cells. However, to date the data obtained in vitro with respect to the production, characterization and biological effects of these polypeptide factors such as sarcoma growth factor has not been extended in vivo in relationship to tumorigenicity. Moreover, although various sarcoma cells produce such factors, it is not known whether carcinoma cells produce similar or different set of agents. The experiments to be conducted should aid in resolving some of these questions.

Proposed Course of Research: To purify these factors from mammary tumors, to relate the production of these factors to the type of cell (preneoplastic or neoplastic), and to determine whether these factors correlate with the endocrine status of the tumor (i.e. hormone dependent or independent).

Publications:

1. Salomon, D.S.: Correlation of receptors for growth factors on mouse embryonal carcinoma cells with growth in serum-free hormone supplemented medium. Exp. Cell Res. 128 (2): 311-327, 1980.

2. Salomon, D.S., Liotta, L.A., and Kidwell, W.R.: Differential response to growth factor by rat mammary epithelium plated on different collagen substrata in serum-free medium. Proc. Nat. Acad. Sci. USA, 78 (1): 382-386, 1981.

3. Zweibel, J.A., Kohn, E. and Salomon, D.S.: Anchorage independent growth conferring factor produced by 7,12-DMBA induced rat mammary adenocarcinoma cells in culture. AACR abstracts, pg. 53, abstract nos. 210, 1981, Washington, D.C.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08252-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Interaction of Growth Factors and Phorbol Ester Tumor Promoters in Cell Growth

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. S. Salomon	Expert	LPP, NCI
Others:	L. Liotta	Expert	LPP, NCI
	D. Bareis	Staff Fellow	LPP, NCI
	E. Schiffmann	Research Biochemist	LDBA, NIDR
	F. Hirata	Research Associate	CP, NIMH
	J. Axelrod	Chief	CP, NIMH

COOPERATING UNITS (if any)

Laboratory of Developmental Biology and Anomalies, NIDR and Clinical Pharmacology Section, NIMH

LAB/BRANCH

Laboratory of Pathophysiology

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The present studies are concerned with the role of hormones and growth factors and their interactions with phorbol ester tumor promoters in relationship to cell growth and differentiation with respect to neoplastic transformation in vitro and in vivo. Several systems are being utilized to study the modulation of growth factor responses and receptor levels by tumor promoters in vitro in cells maintained in serum-free, hormone-supplemented media. A variety of biological endpoints are being examined including cell proliferation, chemotaxis, extracellular matrix (ECM) production, phospholipid metabolism, prostaglandin synthesis and growth factor receptor levels.

PHS-6040
(Rev. 2-81)

Project Description:

The two-stage theory of carcinogenesis has demonstrated that chemical carcinogens (polycyclic hydrocarbons) function as mutagens and produce a series of irreversible events associated with initiation of the transformed state. In contrast, tumor promoters such as phorbol esters function as co-carcinogens by inducing the appearance of tumors following repeated application to cells which have been treated with sub-carcinogenic doses of polycyclic hydrocarbons. Phorbol esters function in the nanomolar range and produce several reversible pleiotypic responses in cells in vitro which resemble those observed upon chemical or viral transformation. These compounds enhance the expression of several transformation specific phenotypic features in already transformed cells and reversibly inhibit cell differentiation in a variety of in vitro systems. The broad range of biological effects produced by these compounds on different cell types at low concentrations are reminiscent of the effects induced by various hormones or growth factors. In fact, several growth factors such as epidermal growth factor (EGF) or hormones such as insulin can synergistically interact with phorbol esters in modulating cell growth. Specifically, EGF can act as a co-carcinogen in mouse skin, while phorbol esters are able to mimic the in vivo biological effect of EGF by causing precocious tooth erruptiion in the neonatal rodent. Moreover, EGF and phorbol esters are able to cross-modulate receptors which mediate the response(s) to these two classes of agents.

Objectives: The effects of phorbol esters on cell growth and differentiation are being examined as these parameters might relate to tumor promotion and progression.

A. Effects of 12-O-Tetradecanoylphorbol-13-acetate (TPA) on Neutrophil Chemotaxis.

Major Findings: TPA inhibits neutrophil chemotaxis in response to specific formylated tripeptides as a result of decreasing the number of chemoattractant receptors on these cells. One effect of TPA is to enhance the activity of a membrane associted phospholipase A_2 ($P\text{-}A_2$). Pretreatment of the cells with glucocorticoids protects the cells against the effects of TPA via the interaction with specific glucocorticoid receptors in neutrophils. Glucocorticoids induce the synthesis of a specific $P\text{-}A_2$ glycoprotein inhibitor (m.w. 40-50,000 daltons). The inhibition of $P\text{-}A_2$ activity by the inhibitor as a result of steroid treatment desensitizes the neutrophil to the effects of TPA. The inhibitor can be isolated from the conditioned medium of steroid-treated cells and can inhibit $P\text{-}A_2$ activity in cells not treated with steroids. Experiments in progress are designed to: 1) characterize the $P\text{-}A_2$ inhibitor; 2) determine the levels of the $P\text{-}A_2$ inhibitor in TPA challenged cells (neutrophils, fibroblasts and embryonal carcinoma (EC) cells) before and after glucocorticoid pretreatment and 3) delineate the role of the $P\text{-}A_2$ inhibitor in modulating neutrophil chemotaxis, chemoattractant binding and responses and binding of other growth factors such as epidermal growth factor (EGF) in EC cells.

B. Effects of Phorbol Esters on the Growth and Differentiation of Mouse EC Cells in Serum-free, Hormone-supplemented Medium.

Major Findings: EC cells are the undifferentiated, malignant stem cells of teratocarcinomas which resemble early embryonic mouse ectoderm. Spontaneous or

retinoid-induced differentiation of EC cells in vitro into several cell types, of which extraembryonic endoderm (END) is the first cell type to arise, mimics the differentiation of the embryonic germ layers of the early mouse embryo. EC cells have recently been grown in a serum-free medium supplemented with epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin (or multiplication-stimulating activity [MSA]), transferrin and Pedersen fetuin (FEIT medium) on substrates such as polylysine or collagen which are necessary for cell attachment and spreading. Growth in FEIT medium is correlated with the presence of specific receptors for EGF, insulin, MSA and transferrin on EC cells. In this medium EC cells differentiate into parietal endoderm cells as evidenced by the appearance of plasminogen activator, the synthesis of type IV collagen, laminin and fibronectin, the presence of a type IV-specific collagenase and the appearance of a laminin degrading activity. Experiments are in progress to determine: 1) the interactions between phorbol esters and growth factors on cell proliferation and the differentiation of EC cells into END cells as these might relate to the modulation of growth factor receptors and ECM production. Recent studies have demonstrated 1) that those phorbol esters which are active as tumor promoters inhibit the binding of [^{125}I]EGF to specific receptors on EC cells; 2) those which are inactive as promoters fail to inhibit EGF binding; 3) the effect is to decrease the number of receptor sites/cell and not an alteration in the affinity; 4) insulin and MSA binding are less affected by phorobol ester treatment; 5) specific phorobol ester receptors have been partially characterized on EC cells; 6) binding to this receptor by various phorbol analogs correlate with the biological activity of these compounds as promoters and with their ability to inhibit EGF binding to its receptor; and 7) EGF can modulate the down regulation of the phorbol ester receptor induced by phorbol esters. (Salomon, D.S.: Tumor promoting phorbol esters inhibit epidermal growth factor binding to receptors on mouse embryonal carcinoma cells, AACR, Abst. #182, San Diego, CA, 1980).

C. Growth of Rat Mammary Epithelial Cells in Serum-free, Hormone-supplemented Medium and Interaction with Phorbol Esters.

Major Findings: Primary cultures of rat mammary epithelial (RME) cells have been grown in FEIT medium supplemented with dexamethasone (dex) on plastic, type I or type IV collagen coated-dishes. The growth of RME cells is regulated by the nature of the substrate upon which the cells are maintained. On plastic or type I collagen, RME cells require EGF and dex for optimal growth while on type IV collagen, growth is unaffected in the absence of either hormone. The differential requirement for EGF and dex on these substrates is not due to changes in the number of receptors for either hormone. However, in the absence of either EGF or dex there is a 50 to 70% reduction in the synthesis of type IV collagen on plastic or type I collagen substrates. Furthermore, in the absence of dex but not EGF a latent collagenase activity which is capable of degrading type IV collagen can be detected in the culture medium following proteolytic activation by plasmin. Although plasminogen activator can be detected in the culture medium, the level of this enzyme is not affected by withdrawal of hormones. These results demonstrate that type IV collagen production is required for RME cell growth and that EGF and dex function as growth regulators by modulating the synthesis and/or degradation of type IV collagen when cells are grown on substrates which are not normal constituents of their basement membrane. Experiments are in progress to determine

the effects of polycyclic hydrocarbons and phorbol esters on these cells and their interactions with EGF and dex on cell growth and ECM production.

D. Effect of Phorbol Esters on Mammary Adenocarcinoma Formation in Carcinogen-treated rats.

Major Findings: A single, direct infusion of several doses of TPA (1-10 μg) into the mammary duct of the third thoracic mammary gland was administered to Sprague-Dawley rats which had been given a single carcinogenic dose of 7,12-dimethylbenzeanthracene (DMBA) 6 mos. prior to the initiation of the experiment. Animals which lacked any palpable mammary adenocarcinomas were chosen to determine if TPA could facilitate the appearance of any latent tumor cells. Ten weeks following TPA administration no significant differences were noted either in the location or frequency of tumor appearance in the control and treated groups. For papilloma production in mouse skin, TPA has to be sequentially administered at regular doses for several weeks before its promoting activity is observed *in vivo*. Experiments are therefore in progress in which similar tumor-free, DMBA-treated rats are being treated bi-weekly with TPA (doses:1-10 g) via s.c. injection into the third thoracic mammary gland for 10 to 15 weeks to determine if these compounds can function as promoters for mammary tumor formation. Future experiments will be designed to determine whether TPA can affect the latency period or modify the frequency of tumor formation in rats administered a sub-carcinogenic dose of DMBA or nitrosomethylurea (NMU).

Significance to Biomedical Research and the Program of the Institute: Tumor promotion by compounds such as phorbol esters is a general phenomenon that has been observed in several tissues *in vivo* including skin, liver, mammary gland, lung, colon and bladder. Phorbol esters directly affect epithelial cells within these tissues by producing a set of reversible phenotypic changes that resemble the malignant or transformed phenotype. Tumor promoters stimulate cell proliferation which in certain instances leads to an inhibition of cell differentiation. Cell proliferation and differentiation are normally controlled by specific hormones or growth factors. It has been proposed that phorbol esters may resemble growth factors or hormones with respect to the type of biological response(s) produced in certain cells to these compounds. Phorbol esters may be mimicing responses to an as of yet unidentified endogenous growth factor(s). Such a situation is not biologically unique since exogenous opiates utilize receptors for endogenous endorphins or enkephalins. The existence of such endogenous compound(s) would have obvious importance not only with respect to their normal function within the animal but also in relationship to tumor promotion and progression. The ongoing studies are utilizing several *in vitro* systems to delineate the interaction(s) of phorbol esters with specific hormones (glucocorticoids and insulin) and growth factors (EGF and MSA) in a defined environment (serum-free medium). EC cells provide a system in which various aspects of cell growth and differentiation can be studied while RME cells or neutrophils provide appropriate models in which cell growth and modulation of certain differentiated parameters can be monitored. The ultimate goal is to utilize these systems to screen for potential endogenous "tumor promoters" which may be related to known or unknown growth factors or hormones. Furthermore, by utilizing RME cell cultures, *in vitro* transformation by carcinogens in the absence or presence of tumor promoters can be tested in a defined environment (serum-free medium) and related to the tumorigenic potential of these treated

cells in the nude (NU-2) mouse and angiogenic activity of these cells to induce neovascularization in the rabbit cornea.

Publications:

1. Hirata, F., Schiffmann, E., Salomon, D.S. and Axelrod, J.S. Glucocorticoid-Induced phospholipase-A_2 inhibitor in rabbit neutrophils. Proc. Nat. Acad. Sci. (USA), 77: 2533-2536, 1980.
2. Salomon, D.S. Correlation of receptors for growth factors on mouse embryonal carcinoma cells with growth in serum-free, hormonesupplemented medium. Exp. Cell Res., 128 (2): 311-327, 1980.
3. Salomon, D.S., Liotta, L.A. and Kidwell, W.R. Differential response to growth factor by rat mammary epithelium plated on different collagen substrata in serum-free medium. Proc. Nat. Acad. Sci.(USA) 78 (1): 382-386, 1981.
4. Sando, J.J., Hilfiker, M.L., Salomon, D.S. and Farrar, J.J. Evidence that specific receptors mediate phorbol ester-enchanced production of T cell growth factor. Proc. Nat. Acad. Sci. (USA) 78 (2): 1189-1193, 1981.
5. Salomon, D.S. Inhibition of epidermal growth factor binding to mouse embryonal carcinoma cells by phorbol esters mediated by specific receptors for phorbol esters. J. Biol. Chem. In press.
6. Sim, R.P., Salomon, D.S., Nylen, M.U. and Pratt, R.M. Tumor promoter TPA mimics epidermal growth factor-induced precocious tooth erruption in the rodent. Photogenesis, Carcinogenesis and Teratogenesis. In press.
7. Salomon, D.S., Liotta, L.A., Rennard, S.I., Foidart, J.M., Garbisa, S. and Yaar, M.A. Synthesis and turnover of basement membrane components by mouse embryonal carcinoma-derived endoderm cells in serum-free medium. Collagen and Disease. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08253-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cellular Mechanism of Action of Phorbol Ester Tumor Promoters

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. J. Sando	Staff Fellow	LPP, NCI
	H. L. Cooper	Chief, Section of Cellular and Molecular Physiology	LPP, NCI
	M. L. Hilfiker	Postdoctoral Fellow	LMI, NIDR
	A. S. Kraft	Research Associate	LPP, NCI

COOPERATING UNITS (if any)

Laboratory of Microbiology and Immunology, NIDR

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cellular and Molecular Physiology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The cellular mechanism of action of phorbol ester (PE) tumor promoters is being investigated in normal human peripheral blood lymphocytes as well as in a mouse T cell line. Both of these cell populations respond to PEs with production of T cell growth factor (TCGF). Specific, saturable, high affinity PE receptors which may mediate the PE stimulated TCGF production have been identified in both systems as judged by appropriate time courses, dose response curves, and structure activity relationships for binding and TCGF production. Current studies are directed at localizing and characterizing the receptor and identifying steps occurring subsequent to PE binding and leading to TCGF production.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Long range goals of this work are to understand the cellular mechanisms involved in tumor promotion in order to determine potential sites for inhibiting the process. Specific objectives for the early part of the work are 1) to identify and partially characterize receptors for PE tumor promoters on normal human lymphocytes and a cultured mouse T cell line (EL4), 2) to further define the steps occurring as a result of the PE-receptor interaction and leading to the production of TCGF, and 3) to determine the steps at which various known or potential modulators of tumor promotion (e.g. glucocorticoids, retinoic acid, prostaglandin synthesis inhibitors) to exert their effects.

Methods:
Cell Sources. Normal human lymphocytes are obtained from heparinized blood by standard Ficoll-Hypaque and adherence techniques. EL4 mouse thymoma cells are maintained in suspension culture.
Receptor Binding. PE receptors are being characterized using a whole cell binding assay involving incubation with ^{3}H-phorbol dibutyrate (PDB) followed by filtration through glass fiber filters to remove free PE. Specific binding is determined by subtracting the ^{3}HPDB bound in the presence of excess unlabeled phorbol myristate acetate from the amount bound in the absence of competitor. Subcellular localization of the receptor is being determined by rupturing the cells and determining the binding in various membrane, cytoplasmic, and nuclear fractions obtained by differential centrifugation.
Assay of PE Responses. TCGF production in normal human lymphocytes is being stimulated by incuabation for 24-40 hr with Concanavalin A plus various PEs in the absence of serum. TCGF production in the EL4 line is stimulated by phorbol esters alone. The TCGF released into the medium is bioassayed for its ability to promote growth (as measured by ^{3}H-thymidine incorporation) of a primary T cell line.

Major Findings: Three basic criteria for the identification of specific receptors have been met for PE binding in normal human lymphocytes: 1) Maximal binding to whole cells is reached within 15 min at 37°C or 2 hr at 4°, long before the production of TCGF by the cells; 2) saturation of the specific binding occurs at a concentration of FPDB (10-30 nM) similar to that required to achieve maximal TCGF production; and 3) competition for PDB binding by structural analogs shows the same order of potency as TCGF production. The specific binding has been demonstrated to be linear with cell number and rapidly reversible. Scatchard plots reveal a single class of sites (approx. 1-3 X 10^{5}/cell) with a Kd of approx. 20 nM. In addition it has been found that the binding at 37° decays during the following 8 h whereas it is stable after reaching maximum at 4°. No evidence for metablism of ^{3}HPDB by normal human lymphocytes has been obtained during incuabation at 37° for at least 20 h and PEs at the concentrations used, do not affect the viability of the cells during this time.

Similar results have been obtained for PE binding in the EL4 cells. Since the EL4 line represents a homogeneous population of cells responding to PEs alone, these results demonstrate that PEs can directly stimulate T cells to produce TCGF. In collaboration with Mary Hilfiker (NIDR), it has been demonstrated that the TCGF production in these cells may be a consequence of receptor mediated inhibition of DNA synthesis. PEs appear to be G_1 blockers in these cells and TCGF may be produced during the G_1 phase of the cell cycle.

Localization of PE binding to crude membrane fractions has been verified in both the normal and cultured cells. In the normal cells, lectins have been found to compete for the binding, suggesting a cell surface location of the receptor. Lectins do not, however, compete for PE binding in the cultured EL4 line or in several human T cell lines.

Significance to Biomedical Research and the Program of the Institute: The results obtained so far are consistent with recent reports from two other laboratories providing evidence for PE receptors in membrane preparation of fibroblasts, mouse epidermal cells and human peripheral blood lymphocytes. The information obtained here considerably expands that obtained in the earlier lymphocyte study. If the receptor is a membrane component, this data may indicate that, analogous to other membrane receptors, the PE receptor is internalized. The existence of specific receptors for phorbol essters also suggests the existence of an endogenous ligand for those receptors. Determining the cellular mechanism of action of this class of tumor promoters may therefore help to explain some natural control mechanisms for cell growth and differentiation as well as to indicate steps in the mechanism which might be affected in disease or pharmacologically to modulate the responses.

Proposed Course of Research: Current work is proceeding in all three areas of the objectives. 1) Attempts are being made to more specifically localize the PE receptor to a specific membrane compartment(s) as well as to examine the reasons for the selective lectin competition for PE binding in normal cells. 2) A variety of known or potential inhibitors of phorbol ester actions in other systems is being examined in the lymphocyte system for possible inhibition of TCGF production and/or inhibition of the "down modulation" of PE receptors occurring at 37°. The types of any effective inhibitors will provide clues about possible mechanisms involved. 3) Direct effects of PEs on several cellular activities suspected from literature or inhibitor studies to be involved in PE action will be examined. These will include effects on various kinase and phospholipase activities.

Publications:

1. Sando, J.J., Hilfiker, M.L., Salomon, D.S. and Farrar, J.J. Specific receptors for phorbol esters in lymphoic cell populations: Role in enhanced production of T-cell growth factor. Proc. Natl. Acad. Sci. USA 78: 1189-1193, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08254-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Acquisition of Angiogenic Capacity by Cell Populations in Neoplastic Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Ziche	Visiting Fellow	LPP, NCI
Others:	P.M. Gullino	Chief,	LPP, NCI
	J. Schlom	Head,	TVDS, NCI

COOPERATING UNITS (if any)

Laboratory of Viral Carcinogenesis, NCI

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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.25	.25	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fibroblasts from Balb/C skin are not angiogenic when cultivated in vitro under standard conditions for 3 generations. Angiogenic capacity is acquired at the 4th passage but neoplastic transformation, defined by tumor formation after transplantation, appears only after the 11th passage. Epithelium from C57/Black mammary gland is not angiogenic; infection with Mu MTV induces angiogenic capacity but the cells are not neoplastically transformed until they are also treated with DMBA. Acquisition of angiogenic capacity in this system indicates increased risk of neoplastic transformation.

PHS-6040
(Rev. 2-81)

Objective: To assess the interdependence between angiogenesis and oncogenesis.

Project Description:

Methodology: (1) Primary cultures of Balb/C skin fibroblasts carried for several transplant generations until a tumor can be obtained upon transplantation in syngeneic host. (2) Each passage was tested with the corneal-angiogenesis assay. (3) 10^{-6}M PMA or 10^{-3}M retinoic acid was added to the culture medium to check whether appearance of angiogenesis could be accelerated or slowed down. (4) Mammary cell line from C57/Black was infected with Mu MTV. Angiogenesis tested before and after infection.

Major Findings: (1) Primary cultures of Balb/C fibroblasts from the skin are not angiogenic for the first three transfer generations. (2) Angiogenic capacity in the rabbit cornea test appears at the 4th generation in about 50% of transplants and in more than 90% at the 7th generation. (3) Sarcoma production by these cells injected subcutaneously in syngeneric hosts was obtained after the 11th transfer generation. (4) An established cell line from mammary epithelium of C57 black is not angiogenic. Injection with Mu MTV induces angiogenesis but not neoplastic transformation. (5) During neoplastic transformation in vitro of these two systems angiogenic capacity appears long before neoplastic transformation. Detection of angiogenesis can predict high risk of neoplastic transformation.

Significance to Biomedical Research and the Program of the Institute: The experiments have established that appearance of angiogenic capacity in cell populations normally deprived of it indicates progression toward neoplastic transformation. This project is part of an attempt to develop means of preventing tumor development by predicting on biopsy material tissues at high risk of neoplastic transformation.

Proposed Course of Research: To complete the project.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08255-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Adenylate Cyclase Activity of Transformed and Normal Fibroblasts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.B. Anderson	Research Chemist	LPP, NCI
Others:	C.J. Jaworski	Chemist	LPP, NCI

COOPERATING UNITS (if any)

M. Olivia Pinkett, Dept. of Zoology, Howard University, Washington, D.C.
Daniele Evain, Unite INSERM 188, 74 Ave., Denfert Rochereau 75014, Paris, France

LAB/BRANCH

~~Laboratory of Pathophysiology~~

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies have been devoted to determining how adenylate cyclase activity might be regulated in fibroblasts growing in culture. The cyclase from Kirsten and Moloney sarcoma virus transformed NRK cells has lost its responsiveness to activation by hormones. Binding studies indicate that the β-receptor for catecholamines is present on these transformed cells, although its affinity for agonists may be somewhat decreased relative to the receptor from control NRK cells. Work is in progress to determine if the mechanism involved in coupling the occupied receptor with the catalytic subunit of the cyclase is defective. Cholera toxin activation of adenylate cyclase appears to require the presence of a plasma membrane-associated reconstituting activity (RA), along with GTP, ATP, and NAD^+. Choleragencatalyzed ADP ribosylation of plasma membrane substrate proteins also requires the presence of RA. These observations indicate that an endogenous membrane-associated factor, along with GTP, may be involved in modulating the ability of choleragen to activate adenylate cyclase. Gonadotropin treatment of CHO cells increases the activity of several enzymes involved in steroidogenesis, indicating that CHO cells in culture might be used to study some of the actions of these hormones.

Project Description:

Objectives: To investigate how adenylate cyclase activity, particularly its hormonal responsiveness, is regulated in normal fibroblasts to, in turn, influence cellular properties under cyclic nucleotide control such as the regulation of cell proliferation.

Methods Employed: Cell culture, standard biochemical analysis of β-adrenergic receptor binding and adenylate cyclase activity, SDS-polyacrylamide gel electrophoresis and radiographic analysis.

Major Findings: Studies have been concerned with elucidating how the hormonal responsiveness of the adenylate cyclase system might be altered and regulated to, in turn, influence cellular properties under cyclic nucleotide control. Incubation of crude NRK membranes with activated cholera toxin in the presence of GTP, ATP, and NAD^+ results in a 10- to 20-fold stimulation of adenylate cyclase activity. Cholera toxin-catalyzed ADP-ribosylation and adenylate cyclase activation of NRK plasma membranes is also dependent upon the presence of a macromolecular "reconstituting activity" (RA). RA is initially associated with crude NRK membrane preparations, but is removed by a low salt buffer wash containing EDTA and DTT. Sephadex G-150 filtration of solubilized RA shows a peak of activity eluting in the region corresponding to a protein with a molecular weight of approximately 13000. RA is eluted from DEAE-cellulose at a salt concentration of 40 to 100 mM KCl. These observations indicate that RA may play a role in mediating the choleragen-catalyzed enhancement of cyclic AMP production.

Previously, we have shown that the properties and activity of fibroblast adenylate cyclase are altered following viral transformation. Transformation of NRK cells by Kirsten or Moloney sarcoma viruses causes a loss in the hormonal responsiveness of the cyclase system. To determine if the β-adrenergic receptor is altered by viral transformation, hormone binding studies were carried out by measuring the specific binding of the β-adrenergic antagonist [^{3}H] dihydroalprenolol (DHA) to membranes prepared from NRK, KNRK, and MNRK cells. Surprisingly, the transformed cells appear to possess a greater number of β-receptors even though the catecholamines do not enhance cyclic AMP production in these cells. However, the receptor affinity for agonists may be decreased in the transformed cells relative to agonist binding noted with NRK cells. Preliminary results indicate that the GTP-dependent mechanism for coupling the occupied receptor signal with the catalytic subunit of the cyclase may be defective.

Previously, we have shown that treatment of CHO cells with gonadoatropic hormones (FSH and hCG) increases the intracellular concentration of cyclic AMP. Recent studies have dealt with a possible steroidogenic effect of gonadotropins on CHO cells. Exposure of CHO cells to gonadotropins is followed by a significant increase in 16α, 17α, and 17β-hydroxylases and 17-20 lyase activities. However, CHO cells do not possess the cholesterol desmolase complex, and gonadotropins do not seem to induce it. Gonadotropic activation of some enzymes involved in the steroidogenic pathway confirm that CHO cells can serve as target cells for studying some of the actions of these hormones.

Significance to Biomedical Research and the Program of the Institute: Cyclic AMP has an important role in influencing numerous properties of normal cells,

including cell growth rate and differentiation. Thus, an understanding of the regulatory properties of adenylate cyclase is of importance. Since viral transformation also produces an altered cyclase system in some cell types, we can better understand how this enzyme is altered by elucidating the regulatory properties of the normal enzyme. Such studies should lead to a better understanding of how this activity is modulated during various stages of cell growth and differentiation and following viral transformation.

Proposed Course of Research: We will continue to elucidate the regulatory properties of the adenylate cyclase system. Studies will be carried out to isolate and characterize the membrane associated "reconstituting activity" (RA) required for choleragen activation of adenylate cyclase. Due to the low amounts of RA obtainable from cultured cells, attempts will be made to find another animal tissue sourcxe of this factor. Other studies will determine if the amount, or activity, of RA is altered under various conditions to, in turn, alter the choleragen activation of cyclic AMP production. Attempts will also be made to further understand the defect in the adenylate cyclase system of transformed cells causing the loss in hormonal responsiveness. Specifically, reconstitution studies will be carried out to determine if the β-receptor, the GTP coupling component, or both, are defective in transformed cells. Also, studies are planned to determine if the GTP-inhibitory process is involved in negating the response to activating hormone.

Publications:

1. Pinkett, M.O., Jaworski, C.J., Evain, D., and Anderson, W.B.: Limited proteolysis eliminates guanine nucleotide inhibition of choleragen-activated adenylate cyclase. J. Biol. Chem. 255, 7716-7721 (1980). 329-332, 1979.

2. Anderson, W.B. and Jaworski, C.J.: Potentiation of hormone-stimulated accumulation of cyclic AMP in cultured fibroblasts by trypsin. Arch. Biochem. Biophys. in press (1981).

3. Evain, D., Anderson, W.B., and Saez, J.M.: Gonadotropin stimulation of pregnenolone metabolism in Chinese Hamster Ovary cells in culture. J. Cell. Physiol. in press (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08256-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Hormones, Calcium and Cyclic Nucleotides in Mediating Cell Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.B. Anderson	Research Chemist	LPP, NCI
Others:	L. Nagarajan	Visiting Scientist	LPP, NCI
	A. Kraft	Research Associate	LPP, NCI

COOPERATING UNITS (if any)

Daniele Evain, Unite' INSERM 188, 74 Ave., Denfert, Rachereau 75014 Paris, France

LAB/BRANCH

~~S. Peter Nissley, Metabolism Branch, National Cancer Institute, NIH~~

SECTION

~~Laboratory of Pathophysiology~~

Office of the Chief

INSTITUTE AND LOCATION

~~NCI, NIH, Bethesda, Maryland 20205~~

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
~~2.0~~	~~2.0~~	~~0~~

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Teratocarcinoma cells in culture offer an in vitro system for studying certain aspects of embryonic differentiation. F9 embryonal carcinoma cells are quite responsive to calcitonin. Exposure of F9 cells to retinoic acid induces differentiation to parietal endoderm cells. Differentiation to the endoderm cell type dramatically alters the hormonal responsiveness of the adenylate cyclase system; the cyclase of endodermal cells exhibits a low response to calcitonin while parathyroid hormone markedly enhances cyclic AMP formation. These variations in calcitonin and parathyroid hormone responsiveness suggest a possible regulatory role for these hormones during embryonic development. Conditions have been established for the growth of F9 cells under serum-free conditions in a medium supplemented with multiplication stimulating activity (MSA), transferrin, and fibronectin. At 100 ng /ml MSA completely replaces the growth requirement for fetal calf serum. Binding studies carried out with [^{125}I]MSA indicate about 55,000 MSA molecules bound per F9 cell with a K_a of 8.2×10^{-9}M. Insulin-like growth factors I and II both compete for binding, while insulin does not compete. These results indicate that MSA is capable of serving as an early embryonic growth factor.

Project Description:

Objectives: To identify agents or conditions which might serve to regulate early biochemical events involved in embryonic differentiation and development.

Methods Employed: Cell culture, standard biochemical analysis of adenylate cyclase and protein kinase activities, hormone binding studies.

Major Findings: Teratocarcinoma cells in culture offer an in vitro system to study some early biochemical events involved in embryonic differentiation and development. Exposure of the F9 line of teratocarcinoma cells to retinoic acid induces the formation of parietal endoderm cells. In turn, treatment of the retinoic acid-induced endodermal cells with dibutyryl cyclic AMP promotes the appearance of neural-like cells. Since the modulation of hormonal responsiveness plays a critical role in the regulation of adenylate cyclase activity, and consequently in the regulation of intracellular cyclic AMP levels, it was of interest to determine any variation in hormonal responsiveness with different stages of differentiation. Basal, GTP- and fluoride-stimulated adenylate cyclase activities show a progressive increase with the retinoic acid induced change of F9 to the endoderm phenotype. Differentiation to the endoderm cell type markedly alters the adenylate cyclase response to calcitonin and parathyroid hormone; the cyclase of endodermal cells exhibits a low response to calcitonin while parathyroid hormone dramatically enhances cyclic AMP formation. Further differentiation of the retinoic acid-generated endodermal cells to a neural-like cell type with cyclic AMP treatment causes the complete loss of calcitonin responsiveness. Studies are in progress to determine if the loss or gain in responsiveness to a particular hormone is due to alteration in the GTP coupling mechanism of the cyclase system or to changes in the hormonal receptors (affinity or number). These results suggest that the embryonic cells might develop at specific stages of differentiation a calcitonin and parathyroid hormone sensitive cyclase system to modulate cyclic AMP and calcium levels, and in this manner regulate specific cellular processes during development.

Calcitonin and parathyroid hormone have both been implicated in modulating calcium flux in different cell systems. Initial studies indicate that calcitonin does enhance calcium accumulation in F9 cells. Changes in intracellular calcium, in turn, might alter activities under calmodulin control and also calcium-dependent protein kinase activities. Preliminary studies indicate that these cells do possess a protein kinase which is stimulated by both calcium and phospholipids, and that the activity of this enzyme changes dramatically with differentiation.

Multiplication stimulating activity (MSA) refers to a family of peptides which have properties similar to the somatomedins, or insulin-like growth factors. Recent studies have shown the MSA levels of fetal rat serum to be 20 to 100-fold higher than the levels found in maternal serum, suggesting a possible role for MSA during embryogenesis. To establish if MSA might serve mitogen function during early embryogenesis, we determined the ability of MSA to support the growth of F9 embryonal carcinoma cells. MSA can serve as a potent mitogen for F9 cells cultured under defined serum-free conditions which include fibronectin as an attachment factor along with transferrin. At 100 ng/ml MSA completely replaces the growth requirement for fetal calf serum. Specific MSA binding to

F9 cells is observed, with Scatchard analysis indicating a single class of MSA binding sites with a K_a of 8.2×10^{-9}. Approximately 55,000 molecules of MSA are bound per F9 cell. Both insulin-like growth factor (IGF) I and II effectively compete for [^{125}I]-MSA binding to F9 cells. MSA also competes for [^{125}I] IGF I and II binding, although higher concentrations of MSA are required to give 50% competition than are required for 50% competition of [^{125}I] MSA binding. Insulin does not compete for either MSA or IGF II binding, although some competition is noted for IGF I binding at high insulin concentrations. Since insulin is an effective mitogen with F9 cells these findings suggest that insulin and MSA exert their mitogenic effects on F-9 cells through independent receptors. It is apparent from these results that MSA is capable of serving as an early embryonic growth factor.

Significance to Biomedical Research and the Program of the Institute: Teratocarcinomas are malignant tumors which are characterized by the presence of a distinctive cell type known as embryonal carcinoma (EC) cells, as well as a variety of differentiated cell types. EC cells lose their malignancy when they differentiate into other cell types in vivo. Thus, it may be possible to modulate the onset of differentiation as a treatment for certain malignant tumors. Further, since the early stages of differentiation in EC cell culture in vitro have certain features in common with the differentiation noted in early mouse embryo, we can gain a better understanding of the process of normal embryonic cell differentiation and learn how malignancy alters this process.

Proposed Course of Research: The development of responsiveness to particular hormones may serve a critical function in regulating the sequence of events leading to differentiation of cells during embryogenesis. Studies will attempt to determine if the loss or gain in response to a given hormone (particularly calcitonin) is due to an alteration in the GTP inhibitory component, to changes in the GTP coupling mechanism, or to changes in the hormone receptors. This will include binding studies to determine changes in the β-adrenergic receptor and calcitonin receptor with differentiation. Other studies will deal with the mechanism of calcitonin action and its ability to alter calcium levels, and activities under calcium and calmodulin control. Included will be studies to define the role of cyclic AMP, calcium, and calmodulin in the differentiation process. This will entail studies with both cyclic AMP-dependent and calcium-dependent protein kinase activities in an effort to establish if these enzymes are altered during differentiation. We will also determine if MSA, or an MSA-like peptide growth factor is being produced by these embryonic cells to serve as a fetal growth promoter. Studies will also be initiated to determine the mechanism of MSA action in promoting the growth of F9 cells, including changes induced at both the membrane level and the nuclear level.

Publications:

1. Binet, E., Evain, D., and Anderson, W.B.: Calcitonin-responsive adenylate cyclase in cultured F9 embryonal carcinoma cells. Exp. Cell Res. (in press).

2. Evain, D., Binet, E., and Anderson, W.B.: Differentiation of F9 embryonal carcinoma cells into endodermal and neural-like cells modifies the adenylate cyclase response to calcitonin and parathyroid hormone. J. Cell. Physiol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08257-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Preparation of Basement Membrane from Human Amnion for use in a New Assay

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. A. Liotta	Expert	LPP, NCI
	R. G. Russo	Visiting Fellow	LPP, NCI
Others:	I. Margulies	Electron Microscopist	LPP, NCI
	U. Thorgeirsson	Visiting Scientist	LP, NCI

COOPERATING UNITS (if any)

Laboratory of Pathology

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tumor cells traverse epithelium basement membranes and collagenous stroma during the transition from in situ to invasive carcinoma and during passage through blood vessel walls and tissue boundaries in the metastatic process. In vivo studies have shown that circulating tumor cells first attach to the exposed basement membrane. Attachment is followed by focal dissolution and penetration by tumor cell pseudopodia. No previous in vitro system has existed for studying this process at the biochemical or ultra structural level. We therefore have prepared whole sheets of intact human amnion basement membrane in an in vitro model of tumor cell invasion. The amnion is placed in a special chamber which allows cells to be placed on one side. Cells which have traversed the amnion are collected and counted on a millipore filter.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The objectives of this project are to prepare whole sheets of intact amnion basement membrane and study the interaction of tumor cells and various normal cells with this substrate.

Methods Employed: Human placentas are obtained following normal delivery and supplied on ice. The amnion membrane is obtained from the chorion by blunt dissection and rinsed in media containing antibiotics. The denuded base-basement membrane can be prepared by a brief treatment with 4% deoxycholate. The whole or denuded amnion is mounted in 3 cm diameter ring-shaped chambers with separate upper and lower compartments. Tumor cells or normal cells are placed on the epithelial surface, the stromal surface or the denuded intact basement membrane surface.

Major Findings: Human amnion is utilized in a new assay for tumor invasion. Electron microscopy and immunohistochemistry studies show amniotic membrane to be composed of a single layer of pavement-type epithelial cells attached to continuous basement membrane overlying an avascular stroma. By immunohistochemistry the basement membrane is shown to contain type IV collagen while the stroma contains type I and III collagens. Direct interaction of normal cells or tumoral cells with the epithelium or the basement membrane or the stroma have been studied independently in vitro. The response to N-formylmethionyl-leucyl-phenylalanine (PMLP) chemoatractant, human polymorphonuclear leukocytes (PMN) penetrated the full thickness of the amnion and were collected and counted. The rate of PMN traversal of the amnion was dependent on the concentration of FMLP (optimal at 10^{-8}) as well as the stage of the FMLP gradient across the amnion. The route of PMN migration was studied by transmission electron microscopy. PMN first attached to the epithelial surface then infiltrated between intercellular junctions. The PMN then penetrated the basement membrane and migrated through the collagenous stroma. Among the tumoral cell lines investigated major evidence of invasion through the amnion membrane was observed with MCF-7 cells (a line derived from a human mammary carcinoma) and one derived from Ewing's sarcoma. Also mouse activated macrophages and mouse melanoma cells demonstrated high invasive potential. Human fibroblasts and mouse normal epithelial cells did not show any invasion through the amnion.

Significance to Biomedical Research and the Program of the Institute: This amnion system has the potential to be used as an assay for human cell invasiveness in vitro. Furthermore it provides a means to study the biochemical and ultrastructural mechanisms involved in the interactions of tumor cells with basement membrane components as well as with the stroma and epithelium.

Proposed Course of Research: a) Ultrastructural and immunohistochemical studies of the normal components of amnion membrane; b) characterize the interaction of a wide variety of human tumor cells, rodent tumor cells and normal cells with the amnion epithelium, denuded basement membrane and stroma; c) to develop a radioactive label method for quantitating tumor cell invasion of amnion basement membrane; d) to study the biosynthesis of basement membrane components by amnion; e) to study the effect of various physiologic enzymes including type IV collagenase on the ultrastructure of the basement membrane; f) to select more invasive or more metastatic cell lines by repeated passages through the amnion.

Publications:

1. Liotta, L.A. Lee, C.W. and Morakis, D.J. New method for preparing large surfaces of intact human basement membrane for tumor invasion studies, Cancer Letters, 11: 141-152, 1980.

2. Russo, R.G., Thorgeirsson, U., Siegal, G.P., and Liotta, L.A. New in vitro assay for cell invasion. Canadian Cancer Society - Proceedings to the symposium on tumor heterogeneity, invasion and metastasis (in press).

3. Russo, R.G., Liotta, L.A., Thorgeirsson, U. and Schiffmann, E. Polymorphonuclear leukocyte migration through human amnion membrane. Jour. of Cell Biol. (in press).

4. Russo, R.G., Siegal, G.P., Lanzer, W.L. and Liotta, L.A. Preparation of radiolabeled human amnion: a new quantitative assay for tumor cell invasion of native human basement membrane. Invasion and Metastasis (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08258-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Characterization of the Collagens Produced by Human Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: L. Liotta	Expert	LPP, NCI
Others: W. Lanzer	Clinical Associate	SURG, NCI
S. Garbisa	Visiting Fellow	LPP, NCI
R. Brundage	Biologist	LPP, NCI

COOPERATING UNITS (if any)

Surgery Branch, NCI

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study is designed to biochemically characterize the collagen produced by several human tumors. Specifically the aims are to study a) the types of collagen produced by various tumors and b) the effect of malignancy transformation on epithelial and chondrocyte collagen biosynthesis.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

1. Establishment of the malignant component of a human chondrosarcoma and breast carcinomas as a continuous line in nude mice.

2. Identification and purification of the collagens produced by human sarcomas.

3. To identify the cell type producing collagen by immunofluorescence.

4. To develop antibodies to the collagens produced and to further characterize them.

Methods Employed: To establish the malignant component of the tumor, a human chondrosarcoma is transplanted into nude mice and serially passaged. Human chondrosarcomas, osteosarcomas, or carcinomas are taken directly from the operating room when tumor cells are still viable. Tumors are then incubated with [^{14}C]proline in the presence of ascorbate, to initiate collagen synthesis, and β-aminopropionitril, to inhibit cross-linking. The collagen then is pH extracted using 2 methods: a) NaCl precipitation at acid and neutral and b) ion-exchange chromatography using a DEAE cellulose column with NaCl gradient. The collagen is then studied by SDS polyacrylamide gel electrophoresis employing pepsin digestion and treatment with bacterial collagenase. To establish the identity of the collagen produced, extracts are subjected to cyanogen bromide cleavage and the subsequent peptides are mapped on polyacrylamide gels. Purified extracts are then injected into rabbits and antibody is collected. Sections of tumor are studied with immunofluorescence using antibodies to types I, II, III and IV collagen.

Major Findings:

1. The chonodroblastic component of a transplanted osteosarcoma produces type II collagen.

2. Human osteosarcoma produces type I collagen.

3. Human protype II collagen is isolated for the first time from the established xenotransplanted chrondroblastic tumor.

4. It is evident that the phenotypic expresion of type II collagen by the xenotransplanted tumor is stable and does not switch to type I collagen synthesis.

Significance to Biomedical Research and the Program of the Institute: The characterization of these tumors can establish what effect malignancy has on the biosynthesis of collagen by chrondrocytes and thus help identify the pathogenesis of these connective tissue tumors. The system using the transplanted tumor can also be used to study the production and stability of proteoglycans and glycoproteins in sarcomas.

Proposed Course of Research: a) to correlate this type of collagen synthesized with the biologic behavior of connective tissue tumors, b) to correlate proteoglycan and glycoprotein synthesis with the biologic behavior of connective tissue tumors.

Publications:

1. Lanzer, W.L., Liotta, L.A., Yee, C., Azar, H., Costa. H. Synthesis of procollagen type II by a xenotransplanted human chondroblastic osteosarcoma. Am. J. of Path. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08259-02 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Role of Type IV Collagenase on Tumor Metastases and Effects of a Specific Antiserum

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. Liotta	Expert	LPP, NCI
Others:	C. Foltz	Research Chemist	LPP, NCI
	W. Lanzer	Clinical Associate	SURG, NCI
	R. Brundage	Biologist	LPP, NCI

COOPERATING UNITS (if any)
Laboratory of Chemistry, NIAMDD; Surgery Branch, NCI

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have already characterized and partially purified a collagenase, neutral metal protease produced by several metastatic tumors, which specifically degrades basement membrane (type IV) collagen. We also have correlated the metastatic potential in vivo of different tumor cell lines with the production of this enzyme. We are trying to obtain an antiserum on this enzyme to better understand the mechanism of activity on the substrate and the effective role of this collagenase in the metastatic process.

Project Description:

Objectives: The objectives of this project are to obtain an antiserum to anti-type IV collagenase and to use it to study the site of action and the mechanisms of activation of the enzyme in vivo, then to check the possibility of inhibition, in vivo and in vitro, the enzymatic activity and the metastatic process.

Methods Employed: Crude collagenase is obtained from serum-free cultures of highly metastatic mouse tumor (PMT sarcoma). The enzyme activity is precipitated with ammonium sulfate and purified by molecular sieve and collagen affinity chromatography. Trypsin activated or inactivated enzyme is applied to gel electrophoresis and the enzyme bands are cut out, minced and injected directly S.Q. into a rabbit. This is followed by booster injections. The specificities of the antibody are be verified by immunoprecipitation. Presence of specific antibodies will be confirmed by immunodiffusion and immunofluorescense experiments on frozen sections. This last procedure is applied to specimens of human tumors obtained at surgery. In addition, the effect of the antiserum on tumor cell metastases in vivo is assayed. After checking the viability of the cells, preincubated with the antibodies, they are injected I.V. in mice to study the possible effect in retarding or preventing their metastatic capacity.

Major Findings: Collagenase activity specific for type IV collagen is already continuously obtained from mice tumors and partially purified in our lab by molecular sieve and Con A-agarose chromatography. Latent collagenase was elaborated by human breast carcinoma cells in continuous culture. Our hypothesis is that this collagenase, which degrades the major structural component of the basement membranes, is an important mechanism that allows the tumor cells to traverse this mechanical and physiological barrier and to reach first the blood circulation and later to leave it and pass on to new tissues.

We have produced antibodies to the partially purified enzyme: 1) after the incubation of the enzyme with the antiserum and precipitation of the complexes, we failed to find enzymatic activity in the supernate; 2) 2 hrs preincubation of PMT sarcoma cells with the antiserum followed by their I.V. injection on to the mice, completely inhibited the formation of pulmonary metastases. The normal rabbit serum did not affect or interfere with the metastatic capacity.

Significance to Biomedical Research and the Program of the Institute: Specific antibody against tumor collagenase may be useful as a diagnostic tool when used in a radioimmunoassay.

Proposed Course of Research: The future course of this research will involve: a) purification of the immunoglobulin fraction from the antiserum; b) radioimmunoassay to quantitate the enzyme; c) active immunization of animals and d) studies on the interaction antibodies-collagenase to have more information on the production and activation of the enzyme.

Publications:

1. Garbisa, S., Tryggvason, K., Foltz, K., and Liotta, L.A. Quantitation of basement membrane collagen degradation by living tumor cells in vitro. Cancer Lett. 9: 359-366, 1980.
2. Garbisa, S., Tryggvason, K., Foidart, J.M. and Liotta, L.A. Assay for radiolabeled type IV collagen in the presence of other proteins using a specific collagenase. Anal. Biochem. 107: 187-192, 1980.
3. Garbisa, S., Liotta, L.A., Tryggvason, K. and Siegal, G. Antibodies to collagenase resistant regions of type IV collagen stain whole basement membrane and cross react with 7S collagen. FEBS Letters, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08261-02 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Copper and PGE During Angiogenesis in vivo

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Ziche	Visiting Fellow	LPP, NCI
Others:	P.M. Gullino	Chief	LPP, NCI
	K. Raju	Staff Fellow	LPP, NCI
	J. Jones		

COOPERATING UNITS (if any)

Analytical Chemistry, F.D.A.

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Evidence has been obtained indicating that: (1) During the angiogenesis process, copper ions are concentrated in the corneal tissue before invasion by new formed capillaries; (2) Animals in copper deficient diet are unable to form new capillaries in the corneal test; (3) Prostaglandin E_1 is able to induce angiogenesis and to concentrate copper ions in the corneal tissue before new formed vessels appear; (4) Indomethacin-treated animals are unable to form new capillaries in the corneal test. Both copper and PGE appear to be indispensable components of the angiogenesis process.

PHS-6040
(Rev. 2-81)

Objective: To elucidate the mechanism of angiogenesis.

Project Description:

Methods Employed: The model used to study angiogenesis is the rabbit cornea. New formation of vessels is evaluated by a steriomicroscope over a period of several days. (1) Corneas treated with PGE, or implanted with neoplastic cells have been removed before new formation of capillaries was evident. Copper content was determined by mass spectrometry. (2) Rabbits were kept in copper deficient diet until copper level in plasma was about half the value before starting the diet. (3) PGE was incorporated into a copolymer pellet. Slow release of PGE, induced angiogenesis in normal rabbits - the same treatment was applied to rabbits treated with endomethacin and implanted with neoplastic cells. Angiogenesis failed to occur. (4) The rabbit treated with endomethacin was implanted with PGE, on the left cornea and neoplastic cells on the right cornea. Only the left cornea produced angiogenesis.

Major Findings: (1) Induction of angiogenesis by PGE, or by neoplastic cells is always preceded by accumulation of copper in the corneal tissue used as a test system. (2) Both PGE and neoplastic cells failed to induce corneal angiogenesis when the rabbits were kept in a copper deficient diet sufficient to decrease copper content to one half the initial value. (3) Treatment of rabbits with endomethacin abolished PGE_1 formation and prevented angiogenesis. PGE_1 added to corneas of rabbits treated with endomethacin produced angiogenesis. (4) Copper ions and PGE_1 appear to be indispensable components of the angiogenesis process.

Significance to Biomedical Research and Program of the Institute: Previous work has indicated that angiogenic capacity is acquired by cells at high risk of neoplastic transformation. By the indentification of the events necessary to induce angiogenesis it might be possible to develop ways of predicting the existence of cell populations at high risk of neoplastic transformation before a tumor is actually developed.

Proposed Course of Research: To complete the project.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08262-02 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Temperature gradients and local perfusion of neoplastic tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P.M. Gullino	Chief, Laboratory of Pathophysiology	LPP,NCI
Others: Rakesh Jain	Professor, Carnegie Mellon Univ. Pittsburg, Pa.	
Flora Grantham	Biologist	LPP,NCI

COOPERATING UNITS (if any)

Carnegie Mellon Univ. Pittsburg, Pa.

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Normothermic tumors showed a circadian rhythm with zenith at midnight and nadir at midday with differences that could reach 3°C. Abdominal tumors were 0.5 to 1.0°C warmer than subcutaneous tumors. The non-uniformity of temperature that existed within normothermic tumors was exaggerated during hyperthermia. No appreciable change in temperature gradients within a tumor was obtained when tumor blood flow was doubled or reduced to one-third. Decrease or increase of blood flow during hyperthermia did not eliminate the sharp temperature gradients. A cold pulse of serum into the tumor efferent artery produced a substantial reduction of tumor blood flow but a small depression of tumor temperature and a very small change in tumor temperature gradients. The data demonstrate extensive anisotropy of blood distribution within a tumor and suggest that thermal diffusion more that convection controls heat transfer within a tumor.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: To define the physiological parameters that appear to control the effects of hyperthermia in tumors.

Methods:

1. Incorporation of thermistors by the growing tumor has been obtained.

2. A new preparation has been developed which permits injection of a pulse of cold serum within the artery afferent to the tumor.

3. A method for continuous recording of tumor temperatures using thermistors has been developed.

Major Findings: (1) The circadian rhythm has the zenith at almost midnight and nadir at about midday, and is independent of changes in ambient temperature. (2) Differences in temperature within a tumor may be more than 2°C. Hyperthermia exaggerates these differences so that temperatures may be lethal in one area but not in another one of the tumors. (3) Extensive modification of blood flow to the tumor does not alter the temperature gradients in both normo- and hyperthermic conditions. (4) Anisotropy of blood distribution within the tumor is a consistent condition. A pulse of cold serum in the tumor artery depresses tumor temperature much less than in normal organs. (5) Thermal diffusion is a predominant event in heat transfer within a tumor, more important than thermal convection depending on blood flow.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the physiological parameters controlling heat diffusion within the tumor improves treatment by hyperthermia.

Proposed Course of Research: To complete the project.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08263-01 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Essential Fatty Acids and Mammary Gland Development and Tumorigenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Barbara K. Vonderhaar	Research Chemist	LPP, NCI
Other: Cristina Pintus	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)

LABORATORY
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is designed to evaluate the developmental patterns of mammary glands of rats and mice fed diets containing different levels of saturated and unsaturated fatty acids. In addition, we wished to establish an atrophied mammary gland similar to that of immature animals but in an "adult" animal. The subsequent impact of manipulation of hormones and dietary lipid intake on mammary tumor incidence is also to be evaluated. Studies include: 1) Examination of the morphology of mammary glands of rats and mice at various ages after being fed EFA-deficient and control diets; 2) determination of the impact of periods of EFA-deficiency in rats on subsequent mammary tumor induction by NMU; 3) evaluation of the effects of reversal from EFA-deficient to control diet on formation of hyperplastic alveolar nodules (HAN) and ultimately on mammary tumor formation in mice; 4) determination of hormonal responsiveness of mammary glands of EFA-deficient mice in organ culture.

PHS-6040
(Rev. 2-81)

Project Description:

Major Objectives: The purpose of these studies was to define the morphologic development of mammary glands of mice and rats placed on an essential fatty acid (EFA) deficient diet for various periods of time. Subsequent formation of hyperplastic alveolar nodules (HAN) and mammary tumors was to be assessed. The hormonal responsiveness of the mammary glands from EFA-deficient mice was to be assessed in organ culture.

Methods Employed: Inbred Fisher 344 and C3H/HeN ($MMTV^+$) mice were used. Pregnant animals were placed on either a control of EFA deficient diet at mid-term. Female offspring were maintained on the appropriate diet until 3 mon. of age when experiments were begun involving dietary and hormonal manipulations. Morphology of mammary glands was examined by hematoxalin staining of whole mounted preparations. Hyperplastic alveolar nodules (HAN) were identified and counted with the aid of a dissecting microscope. An organ culture system using chemically defined serum-free medium was employed for studies on hormonal responsiveness in vitro. The experimental diets were similar in 95% of total composition. The remaining 5% of the control diet was composed of corn oil. The EFA-deficient diet contained 5% triglycerides resynthesized from fatty acids of 8-14 carbon chain length purified from hydrolyzed coconut oil. The diets were checked for lipid content, lipid profile and fatty acid composition by gravimetric analysis, thin-layer, and gas-liquid chromatography respectively and their compositions confirmed as correct.

Major Findings: Rats which were placed on either the control(C) or EFA-deficient (D) diet in utero were examined at various ages to determine the effect of the deficiency on survival, body weight and general health, body fat deposition and mammary gland development. During the first 7 months of life both the C and D groups had over 95% survival. Beyond 9 mon. of age (in the tumor study) D animal began to show higher mortality rates due to increased severity of the deficient state. At 3-4 mon, the average body weight of autopsied animals in the D group was 80-90% of the C group. By 6-7 months the D group weighed only 65-70% of the C group. This observation reflected lower food consumption by D rats resulting in a general deterioration in the health and body fat stores of the D group with time. The #4 and 5 abdominal mammary glands were removed from animals at 4, 7 and 9 mon. of age, weighed and whole mounted for morphological examination. At 4 mon. the glands from D and C animals weighed 0.773 gm and 1.109 gm respectively. At 7 mon. the average weight of the glands was 0.808 gm (D) and 1.5 gm (C) and at 9 mon, 0.352 (D) and 1.821 gm (C). Uteri of the 9 mon. old animals weighed an average of 209.7 mg (D) and 482 mg (C). The whole mounted glands were examined in each group. At 4 mon. of age, little difference in morphology was observed between the groups although the degree of development was less uniform in the D than in the C rats. In both cases the epithelial component filled out the fat pad with multiple branched ducts with growing end buds. Most control glands showed some alveoli. Alveoli tended to be less frequent in glands of D rats. At 9 mon. of age all glands of C anmals had continued to develop with many alveoli. Glands from D animals remained relatively devoid of alveoli.

Rats on the 2 diets were then divided into 3 age groups (60, 110, 165 days old) at which time NMU treatments were begun. A portion of D animals in each

age group were switched to control diet (D-C) 48 hr prior to the onset of NMU treatment. This switching in diet was expected to initiate epithelial cell growth and possibly render the dormant gland more susceptible to NMU carcinogenesis. These tumor studies are still in progress.

C3H/HeN mice placed on the EFA-deficient diet in utero developed a more severe deficiency more rapidly. Of the mice received by us at 3 mon. of age, only 50% were able to survive an additional month on the deficient diet. D mice 8-11 wks old weighed only 50-60% of controls. This difference continued even with those few D mice able to survive 8 mon. When 3 mon. old D mice were switched to C diet (D-C) significant increases in body weight were seen within one week. After 1 month of reversal of diet the animals' body weights were not significantly different from C animals of a similar age. Even at 3 mon. of age D animals' body fat was nearly non-existent. Uteri of these animals weighed an average of 8.8 mg compared to 84.0 mg in C animals. The average weight of the #4 mammary gland of 3 mon. old D mice was 34 mg vs 107 mg for C mice. One month after reversal from D to C diet, the uteri weighed 79.9 mg and the mammary glands 137 mg which is not significantly different from the weight of these organs in 4 mon. old C mice. Whole mounts of the glands of these animals gave the following results. The epithelial component of mammary glands of 3 mon. old D mice consists of a short outgrowth from the nipple with little ductal branching and no alveoli. At most, this outgrowth fills 1/4 of the sparse fat pad. In contrast, the fat pad of the 3-4 mon. old C animals is completely filled with highly branched ducts and frequent alveoli. The D-C mice after 1 mon. of reversal had visibily increased the mammary fat pad size and the epithelial component underwent a burst of growth. This resulted in many branched ducts which filled the fat pad. Few alveoli were present and frequently the ducts appeared distended or dilated.

A group of D-C female mice were placed with breeder males 6 weeks after dietary reversal. Of the 12 females in the study, 6 became pregnant between 13 to 38 days after mating. The experiment was terminated after 121 days.

Mammary glands from 3 mon. old D and C mice and 4 mon. old D-C mice were placed in organ culture in the presence of insulin, hydrocortisone and prolactin. Both C and D-C animals' glands wre able to synthesize milk proteins in response to the hormones. Glands from D mice made neither caseins nor α-lactalbumin.

Development of "HAN" was examined in C and D-C virgins at 6 mon and 9 mon of age. All C animals had HAN at both ages (19/19 and 11/11 respectively). In the D-C groups 11/17 had HAN at 6 mon. and 11/14 at 9 mon. At 6 mon. the average number of HAN for C mice was 9.6 (range 2-27) and 5.0 (range 1-11) for D-C. At 9 mon. the C group had 20.9 (range 2-44) and D-C had 6.6 (range 1-29). The outgrowths of the latter group frequently were associated with distended or dilated ducts and resembled ductal hyperplasias rather than typical HAN. A study on mammary tumorigenesis in these dietary groups is in progress.

Primiparous animals were also examined for alterations in mammary gland morphology, appearance of HAN and subsequent tumor incidence as a result of the EFA-deficient diet. Mid-pregnant mice were placed on either the C or D diet and maintained on that diet throughout pregnancy, lactation and for 12 weeks after pups were weaned. At this time several animals in both groups were killed and mammary

glands whole mounted. All animals examined had HAN. The average number of HAN for C mice was 32.6 (range 10-95) and for D mice was 16.1 (range 3-38). In the D group dilated ducts and ductal hyperplasias were often seen.

Because of poor survival beyond 12 wk, the D primiparous mice were switched to control diet at this time. Assessment of tumor incidence in the C and D-C groups is currently in progress.

Significance to Biomedical Research and the Program of the Institute: Epidemiological studies have shown a strong positive correlation between per capita fat consumption and the incidence of human breast cancer in various countries. Elevated dietary fat has been shown to facilitate development of both spontaneous and carcinogen-induced mammary tumors in rodents. Elevated dietary lipids have been shown to alter prolactin secretion and clearance in rodents. Prolactin may play a key role in initiation of mammary tumorigenesis. In addition unsaturated fats may act directly on the mammary gland to affect the growth, development, and hormonal responsiveness of the epithelial cells. In the U.S. recent studies have indicated that while the level of consumption of animal fat has been decreasing, increased consumption of vegetable fat, high in unsaturated fatty acids, has occurred. Thus we are designing studies to help to understand the impact of the unsaturated, essential fatty acids on mammary development, hormonal sensitivity and subsequent tumorigenesis.

Proposed Course of Research: The basic study of EFA-deficiency on mammary tumor incidence in rats and mice will be completed. Work will begin to assess the effects of alterations in the quality of dietary lipid, rather than quantity, on mammary gland development and tumorigenesis. This will involve use of diets containing 5% vegetable or 5% animal fat in place of corn oil. The impact of the change in dietary fat on mammary fat pad composition will be assessed as well as the impact on the dynamics of epithelial cell growth. The effects of hormonal imbalance during the period of growth after dietary reversal will be assessed in terms of mammary morphology, fat pad composition and subsequent tumorigenesis.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08264-01 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotide and Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y.S. Cho-Chung	Chief, Cellular Biochemistry Section	LPP,NCI
Others:	H. Huang	Expert	LPP, NCI
	T. Hasuma	Visiting Fellow	LPP, NCI
	T. Clair	Chemist	LPP, NCI
	C. Shepheard	Biologist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cellular Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	0.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Single intubation of DMBA to 50 day old rats produces mammary carcinomas at 100-120 days of age. The tumor production was preceded by changes in the cAMP system: Within 24 hr post DMBA intubation, the intracellular cAMP level and adenylate cyclase activity increased while cAMP-dependent protein kinase activity decreased in the mammary gland. DibutyrylcAMP orally administered blocked these changes in the cAMP system and suppressed tumor production. Thus, aberration of the cAMP system is an early event in DMBA-induced carcinogenesis and exogenous DBcAMP blocks this event.

PHS-6040
(Rev. 2-81)

Project Description:

Methods Employed:

i. DMBA intubation.
Sprague-Dawley female rats (50 days old) were given a single intubation of 7,12-dimethylbenz(α)anthracene (DMBA) (20mg in 1ml sesame oil).

ii. DBcAMP administration.
DBcAMP (2mg/rat, 3 times/week) was given orally beginning 7 days prior to DMBA intubation.

iii. Cyclic AMP level: intracellular cAMP content was measured by the radioimmunoassay using the acetylation procedure exactly as described by Collaborative Research Inc.

iv. Adenylate cyclase assay: Adenylate cyclase activity was measured by the method of Krishna et al.

v. Photoaffinity labeling of cAMP-binding protein: The photoactivated incorporation of 8-N_3-[^{32}P] cAMP into cytosol or membrane proteins was performed by the method of Pomerantz et al.

vi. Protein kinase assay: The activity was measured by measurement of the incorpation of ^{32}P from γ-labeled ATP into histones ± 10^{-6}M cAMP.

Major Findings

I. The changes in the cAMP system preceded tumor production following DMBA intubation

1. Within 24 hr post DMBA intubation, the intracellular cAMP level, adenylate cyclase activity and NAD-dependent ADP-ribosyl transferase activity markedly increased while cAMP-stimulated protein kinase activity decreased.

2. The decreased of kinase activity reflected a change in the molecular species of cAMP-binding protein, the regulatory subunit of cAMP-dependent protein kinase.

3. Control mammary gland cytosol contained a single cAMP-binding protein of 56,000-daltons that incorporated the photaffinity label, 8-N_3-[^{32}P]cAMP. Within 24 hr post DMBA intubation, the 56,000 dalton protein decreased and the 39,000-dalton protein (Proteolytic fragment of the 56-000 daltons) appeared in the mammary gland cytosol.

4. These changes in the cAMP system did not occur in other tissues, suggesting a link between the cAMP system and carcinogenesis in the gland.

5. In order to examine whether DMBA can directly act on mammary gland, the slices of mammary gland were incubated in vitro with DMBA at 30°C for 15 min. DMBA in vitro, indeed elicited the changes in the cAMP system of mammary gland similar to that seen with DMBA intubation in vivo.

II. Exogenous DBcAMP blocks DMBA-induced carcinogenesis

1. A single intubation of DMBA to 50 day old rat produced mammary carcinomas in 76% of rats at 100~120 days of age.

2. Orally administered DBcAMP beginning 7 days prior to DMBA intubation delayed the time of tumor appearance and greatly reduced tumor production: only 15% of rats produced tumors by 180 days of age. When DBcAMP was given 3 days post DMBA intubation, however, the suppression of tumor production was only 50%.

3. Concomitant with the suppression of tumor production by DBcAMP was the blocking of the DMBA-induced changes in the cAMP system of the mammary gland.

4. DBcAMP also blocked the effect of DMBA in vitro. When the slices of mammary gland were preincubated with DBcAMP+cAMP-dependent protein kinase type II prior to the DMBA incubation, the changes in the cAMP system of the mammary gland due to DMBA incubation were prevented.

These results suggest that aberration of the cAMP system is an early event in DMBA-induced carcinogenesis and that exogenous DBcAMP blocks this event.

Significance to Cancer Research and the Program of the Institute: (NCP Objective #6, approach #3) These studies contribute to the understanding of the molecular mechanism of carcinogenesis. Our studies clearly show the involvement of the cAMP system in the mammary carcinogenesis. The suppression of mammary carcinogenesis found with orally administered DBcAMP may be of great significance in the prevention of mammary cancer in humans.

Proposed Course of Research: To extend the investigation on the mechanism of the action of cAMP in carcinogenesis, the following proposals are made: 1) Assess the subcellular localization of [^{14}C]DMBA and [^{3}H]DBcAMP following their administration in vivo and in vitro to detect the site(s) of their antagonistic action, 2) Examine DNA-adducts following DMBA intubation, 3) Assess whether phosphorylation of plasma membranes or nuclear proteins takes place with DMBA $\pm$ DBcAMP intubation. 4) Examine the in vitro translation of poly A RNA isolated from mammary glands following DMBA $\pm$ DBcAMP intubation.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08265-01 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of a Type V Collagenolytic Enzyme

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lance Liotta	Expert	LPP, NCI
	William L. Lanzer	Clinical Assoc.	LP, NCI
	S. Garbisa	Visiting Fellow	LPP, NCI
	T. Kalebic	Visiting Fellow	
	R. Brundage	Biologist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A neutral metal protease has been identified which cleaves native type V collagen under conditions where pepsinized type IV collagen or the interstitial collagens are not significantly degraded. The enzyme is secreted into the media of cultured M50-76 reticulum cell sarcoma (malignant macrophages) and leiomyosarcoma tumor cells. Biosynthetically labeled type V collagen prepared from organ cultures of human amnion membrane is used for a routine assay of type V collagenolytic activity. The partially purified enzyme a) exists in a latent form requiring trypsin activation for maximum activity; b) has a molecular weight estimated by molecular sieve chromatography of approximately 80,000 daltons; c) is inhibited by EDTA but not phenylmethylsulfonyl fluoride; and d) produces specific cleavage products of both A and B collagen chains.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To identify and characterize collagenolytic activity for type V collagen and to purify this enzyme.

Methods: Serum free culture media reticulum cell sarcoma and a human leiomyosarcoma is precipitated with ammonium sulfate and the resultant proteins are dialyzed and chromatogaraphed on a molecular sieve column and further purified by high pressure liquid chromatography. Enzyme activity is assayed using polyacrylamide gel electrophoresis of cold type V collagen and trichloroacetic acid/tannic acid precipitation of ^{14}C labeled type V collagen digestions products. Further studies of purified enzyme are performed using isoelectric focusing.

Major Findings: After surveying a number of tumor cell lines for proteases we have now identified a neutral type V collagen degrading metal protease for the first time. Under usual digestion conditions this enzyme is selective for type V collagen and produces specific large molecular weight cleavage products for both αA αB and αC chains. The enzyme exists in latent form requiring activation by trypsin.

Significance to Biomedical Research and the Program of the Institute: A type V collagenolytic enzyme has been identified and purified for the first time. Since type V collagen may be pericellular in location, cell migration may abe associated with type V collagen turnover. Moreover, the presence of a type V degrading enzyme in tumor cells may in part have some influence on these cells ability to invade tissues and on their metastatic organ distribution.

Proposed Course of Research: a) To develop antibodies to the type V collagenase to see if this activity can be inhibited, b) to survey other tumor cell lines for this enzymatic activity, c) to further characterize and purify this enzyme with other biochemical methods, d) to utilize this enzyme in the study of the molecular structure of type V collagens.

Publications:

1. Liotta, L.A., Lanzer, W.R. and Garbisa, S. Identification of a type V collagenolytic activity. Biochem. and Biophys. Res. Comm., vol. 98, no. 1, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08266-01 LPP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Degradation of Laminin and Determination of Its Molecular Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lance Liotta	Expert	LPP, NCI
Others:	William L. Lanzer	Clinical Associate	SURG, NCI
	Nagasawara Rao		
	R.G. Brundage	Biologist	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Highly purified α-thrombin, plasmin and urokinase were incubated with laminin, type IV collagen and type V collagen. At 25°C (1:100 enzyme to substrate ratio on a weight/weight basis) α-thrombin selectively degraded the β chain of the native laminin, whereas plasmin degraded both the α and β chains. The specific limited cleavage fragments of laminin produced by α-thrombin and plasmin retained the ability to mediate binding of epithelial cells to type IV collagen. These serine proteases failed to degrade native type IV or V collagen under identical experimental conditions. At 35°C type V collagen, but not type IV collagen, was partially cleaved by both α-thrombin and plasmin. Urokinase failed to degrade any of the substrates. Specific cleavage products are used for structural and immunological studies.

PHS-6040
(Rev. 2-81)

Objectives:

1. To determine the susceptibility of native laminin to degradation by serine proteases.

2. To use the degradation products of laminin by these proteases to further study the structure and biological function of laminin.

Project Description:

Methods: Highly purified α thrombin, urokinase or plasmin were incubated at 35°C M 25°C for varying time periods with laminin, type V and type IV collagen. Laminin was extracted from EHS mouse sarcoma tissue by salt extraction and DEAE cellulose and agarose B50 Gel-A-5m column chromatography. Purified laminin was also iodinated with I^{125} and digestion products were run on polyacylamide gel electrophoresis. Digestion products were also run on a B10 Gel-A-1.5 column in order to separate by molecular weights digestion products.

Major Findings: α thrombin selectively cleaves the 400 KD(B chain) of laminin whereas plasmin cleaves both the 400 KD and 200 KD (α chain). At 25°C neither type IV nor type V collagen was degraded by any of the proteases. The combined cleavage products of non-denatured laminin digestion (25°C) by either plasmin or thrombin retained the native molecules ability to mediate attachment of epithelial cells to type IV collagen. None of the substrates were degraded by urokinase.

Significance to Biomedical Research and the Program of the Institute: (1) Degradation of laminin may play a role in endothelial migration during wound healing or vascularization since migration may involve a series of sequential binding events. (2) Degradation of laminin may also facilitate passage of: immune cells and tumor cells through basement membranes. (3) Using these degradation products will allow study of the structure of laminin and may produce antibodies to various components to the molecule.

Proposed Course of Research: (1) To separate the chains by column chromatography after selective cleavage. (2) To use antibodies against these separated cleavage products to study the structure of laminin and determine where the attachment site is located. (3) To produce antibodies to the laminin attachment site and investigate it . Tumor cell attachment can be blocked by these antibodies.

Publications:

1. Liotta, L.A., Goldfarb, R., Tenanova, V. Cleavage of laminin by thrombin and plasmin: Alpha thrombin selectively cleaves the beta chain of laminin. Thrombosis Research (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08267-01 LPP

PERIOD COVERED

October 1, 1980 to September 30, 1981 (terminates July 31, 1981)

TITLE OF PROJECT (80 characters or less)

Hormonal Status and Gene Expression in Normal and Neoplastic Rat Mammary Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Horn	Staff Fellow	LPP,NCI
Other:	S. Parmenter	Biologist	LPP,NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

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NCI, NIH, Bethesda, Maryland 20205

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1.5	1.0	.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In conjunction with P. Qasba, A. Dandekar and S. Safaya: Expression of milk protein and other genes in different hormone environments has been examined in normal and neoplastic rat mammary tissues. We studied the effects of ovariectomy during pregnancy, perphenazine administration, and a mammotropic hormone secreting pituitary tumor. The general pattern of gene expression was monitored by translating total poly A(+)RNAs in a wheat germ protein synthesis extract and subjecting the translation products to one- and two-dimensional polyacrylamide gel electrophoresis. Specific poly A(+)RNAs were monitored by filter hybridization to probes made from cDNA clones for abundant lactating mammary gland poly A(+)RNAs. Expression of α-lactalbumin was examined in detail using immunological methods and by gel electrophoresis.

PHS-6040
(Rev. 2-81)

protein and three other low molecular weight species that have not yet been characterized.

This study was extended using filter hybridization to estimate abundances of various specific RNA sequences. Replicate filters were prepared that contained 50, 10 and 5 ng of lactating mammary gland RNA, RNAs from the ovariectomized and sham-operated pregnant mammary glands and from unoperated 14- and 16-da pregnant glands. These filters were hybridized individually to each of the 6 cDNA clone probes. We found that after ovariectomy α-lactalbumin sequences increased most dramatically (at least 10-fold). γ-casein and wp-protein sequences increased about 4-fold, and α- and β-casein and k-protein RNA sequences did not appear to change (<2-fold increase).

Perphenazine treatment of virgin S-D rats resulted in a 2-fold increase in α-lactalbumin content within the first day as monitored by radioimmunoassay. By 4 days of treatment α-lactalbumin content had increased 10-fold. Filter hybridization studies of 100 ng each of the poly A(+)RNAs showed that α-lactalbumin sequences increased two-fold also within 1 day of perphenazine treatment and increased somewhat more by day 4. If the rats were ovariectomized just before the start of perphenazine treatment, α-lactalbumin sequence content increased even further. Sequences corresponding to Wp-protein, k-protein, and γ-casein were barely detectable in the RNAs of virgin rats. However, perphenazine treatment led to a large increase of k-protein sequences and about two-fold overall increases in the other sequences. Ovariectomy increased α-lactalbumin and γ-casein sequences to levels comparable with lactating tissue while α-and β-casein sequences were not further affected.

Effect of MtTW10 Growth of the MTW9 tumor requires co-transfer of the MtTW10 pituitary tumor. Growth of the host mammary gland is also stimulated. The pattern of gene expression in the host tissue differs from that of the mammary tumor in that the host RNA contains significant amounts of k and wp protein sequences. α-Lactalbumin sequences are most sensitive to the time of exposure to MtTW10. At three weeks after tumor transfer, no α-lactalbumin sequences are present but by four weeks, its RNA levels increase. Ovariectomy at three weeks doesn't affect the increase at 4 weeks. MTW9 doesn't become palpable until 4 weeks after tumor transfer so it isn't known whether α-lactalbumin sequences are present in the tumor at earlier times.

Developmental analysis. Poly A(+)RNAs from normal mammary glands and livers of virgin, pregnant, and lactating rats had been isolated and examined by cell-free translation as reported last year (P. Qasba, PI). These have now also been analyzed by filter hybridization. One filter contained 100 ng per spot of mammary gland several RNA preparations from virgin, days 5-20 of pregnancy, days 5-19 of lactation, RNAs from livers and from MTW9, MCCLX and DMBA tumors. Replicate filters were hydridized individually with each of 6 cDNA clone probes: p18 (α-lactalbumin), p52 (Wp protein), p94 (κ-protein), p305 (γ-casein), p303 (42K casein), and p530 (25K casein). Each sequence showed a unique pattern during functional differentiation of the mammary gland. In all cases, the barely detectable signal observed in liver RNA was assumed to be non-specific (although liver may have

Methods:

14 day pregnant Sprague-Dawley rats were ovariectomized or sham-operated. 40 h later their mammary glands were removed for analysis. Virgin Sprague-Dawley rats were injected daily for 0 - 4 days with perphenazine. In a second experiment, virgin rats were ovariectomized then injected daily for three days.

Wistar/Furth virgins were implanted with the MtTW10 mammotrophic hormone secreting pituitary tumor. At three and four weeks the mammary glands were removed. At three weeks a group of rats was ovariectomized and 40 h later the mammary glands were removed.

In addition, several mammary tumors were examined, namely MCCLX, MTW9 and MTW9A. In progress are similar studies of individual NMU-and DMBA-induced mammary tumors.

In some cases radioimmunoassay for α-lactalbumin was performed.

Poly A(+) RNAs were extracted from all of these tissues. In most instances, portions of the poly A(+)RNAs were analyzed by cell-free translation and gel electrophoresis.

The RNAs were also fixed to nitrocellulose filters and hybridized with radioactive probes of 6 cDNA clones: p18, p52, p94, p305, p303 and p530 (reported by P. Qasba et al) using a sensitive new "dot blot" hybridization technique (P.S. Thomas PNAS 77: 5201-5205,1980). Filters were hybridized, washed, and exposed to X-ray film. The autoradiograms constitute the data. Several kinds of controls were performed. 1) Liver RNA, which was defined to have no milk protein RNA sequences, was spotted at the same concentration as the mammary gland RNA (standard =100 ng per 1 µl spot). Except at long exposure times, no signal was detectable in the liver RNAs. 2) Decreasing concentrations of mammary gland RNAs were spotted and after appropriate hybridization and exposure times, spot intensity after autoradiography was dependent on the RNA concentration. 3) Hybridization was performed at 42°C in 50% formamide, but the filters were washed at high stringency (68°C, 0.1X SSC, 0.1% SDS). Background was essentially undetectable.

Major Findings:

The effect of ovariectomy on gene expression in the mammary glands of pregnant rats was examined in detail. Others had reported that lactose accumulates rapidly and dramatically within 40 h post-op and α-lactalbumin activity increases 30-fold from barely detectable levels by 30 h post-op. Casein increases about 2-fold from already significant levels. Using radioimmunoassay, I found a 60-fold increase in α-lactalbumin content 40 h post-op compared to barely detectable levels in sham operated controls, although total protein content didn't change signficantly. Total poly A(+)RNA translation activity did not change, but there was a large increase in a protein product having an apparent molecular weight of about 21,000 daltons that was specifically precipitated with α-lactalbumin antiserum. Analysis of total translation products by 2-dimensional gel electrophoresis showed that several other spots, besides the one corresponding to α-lactalbumin increased dramatically after ovariectomy. These include translation products corresponding to γ-casein, Wp-

sequences that cross-hybridize to p94). Of all the sequences, α-lactalbumin (p18) had the most unusual pattern, showing a transient increase in RNA abundance between days 8-12 of pregnancy. All of the other sequences began to increase at different times between days 5 and 10 of pregnancy and continued to increase gradually with further increases during lactation. MTW9 and MCCLX RNAs both showed significant abundance of α-lactalbumin-specific sequences while the primary DMBA tumor tested thus far showed low but detectable amounts of this sequence. MCCLX and MTW9 had low levels of p94 and p52 sequences but contained high levels of p303, p305 and 530. p52, p305 and p530 sequences were apparently absent in the DMBA tumor.

In progress: Preliminary results using filter hybridization indicate that MTW9A tumors growing in virgin rats contain little if any of the bundant lactating mammary gland sequences. Tumor-bearing rats have now been treated with perphenazine (which might be expected to increase α-lactalbumin levels), but the RNAs have not yet been analyzed. Also, individual NMU tumors of perphenazine and control treated rats are being examined as are individual DMBA tumors.

DNA has been extracted from many of the mammary tisues described. These will probably be analyzed by others to determine if and how organization of the particular lactating mamamry gland-specific sequences change with hormonal status of the tissue.

Significance to Biomedical Research and the Program of the Institute: I think the filter hybridization procedure will prove to be extremely valuable and practical for screening tumors in comparison with normal mammary tissues. The technique is extremely sensitive and fairly easy to perform (certainly as compared with radioimmunoassay and CRot analysis). It utilizes small amounts of RNA and the filters are reusable and thus far have been extremely stable.

Comparison of a battery of specific RNA sequences will enable us to establish normal patterns of gene expression and to determine the extent of hormonal coordination of these patterns. The data obtained from a similar screening of a variety of transplantable and primary tumors will allow us to determine what changes have occurred in their patterns of gene expression and whether there is a common pattern in these changes

Proposed Course of Research: I will begin studies in cooperation with Dr. B. K. Vonderhaar on the role of methylation and hormone responsiveness in mouse mammary gland. Lactogenic hormone binding is known to increase when mouse mammary gland membrane methylation (especially of phospholipid) increases. I will assay binding of other hormones, e.g. insulin and epidermal growth factor to determine whether increased membrane methylation affects binding of all hormones in the same or different ways. Inhibitors of methylation, such as 5-azacytidine and 2-deoxyadenosine apparently alter gene expression in trend leukemia and 3T3 cells. We will test these drugs, and other methylation inhibitors such as methotrexate, to see how they affect α -lactalbumin synthesis in mammary explants, and hormone binding on cell membranes as a first analysis. Such a study could provide much insight into understanding how cells become unresponsive to chemotherapeutic agents such as methotrexate.

Publications:

1. Qasba, P.K., Dandekar, A.M., Sobiech, K.A., Nakhasi, H.L, Devinoy, E., Horn, T., Losonczy, I., and Siegel, M. Milk protein gene expression in the rat mamamry gland, Critical Reviews of Food Sciences and Nutrition, in press.

LABORATORY OF MATHEMATICAL BIOLOGY

SUMMARY

October 1, 1980 through September 30, 1981

Summary Report

The activities of the Laboratory of Mathematical Biology (LMB) fall into several broad areas: membrane biophysics, immunology, macromolecular configurations, kinetics of metabolic systems and computational and modeling methodology. Most of the work is theoretical, but experiments are also carried out in the laboratory. Much of the theoretical work and modeling is done in collaboration with experimental groups at NIH and elsewhere.

The studies in the section on Membrane Structure and Function (Blumenthal, Weinstein, Klausner, Van Renswoude, Kempf) focus on questions of assembly, topology and conformation of proteins in membranes. Two approaches are followed; one physical chemical, the other molecular biological. The physical chemical approach makes use of lipid model membranes. We measure 1) release of carboxyfluorescein, a water soluble fluorescent dye, encapsulated in lipid vesicles, and 2) conductance across planar black lipid membranes (BLMs) to monitor interactions of proteins with membranes. We use spectroscopic techniques (fluorescence, circular dichroism) to study changes in protein conformation upon interaction with lipid, and BLM techniques to approach questions of membrane protein topology. We also study the dynamics and organization of membrane lipids, since interfaces between domains of lipids with different packing might serve as sites for insertion of membrane proteins. The following systems are investigated: 1) Interactions of serum lipoproteins (apo-A1, apo-A2) and of tubulin with lipid vesicles at the lipid phase transition. The nature of the recombinants formed, and their "physiological" roles are being examined. 2) The effect of the membrane potential and of ligand on the disposition of membrane proteins. Conductance changes in BLMs caused by lipid perturbations induced by certain membrane proteins are used as a probe for the disposition of the protein. The hepatic asialoglycoprotein receptor is translocated across a bilayer under the influence of a trans-positive membrane potential in the presence of ligand. A portion of mellitin is translocated under the influence of a trans-negative membrane potential. We have carried out a theoretical analysis on the way electrostatic forces operating on charge clusters, found in transmembrane proteins, may determine the final orientation of the protein. 3) Conformation and proton pumping of bacteriorhodopsin are studied in reconstituted vesicles. Fluorescence energy transfer from bacteriorhodopsin tryptophan to anthrostearic acid probes inserted into vesicles allows us to map the spatial distribution of tryptophans, and model the conformation of the protein in the membrane. Studies of the detailed proton pumping kinetics allows us to model the processes involved in pumping of protons. 4) To understand the mechanism whereby secreted proteins are translocated across the endoplasmic reticulum membrane we are studying ovalbumin. When ovalbumin is denatured in vitro it does not return to its native form upon renaturation but assumes a new form called OAR. It has been characterized as distinct from native ovalbumin by spectroscopic, chemical, and immunological techniques. OAR has definite hydrophobic characteritics including interaction with lipid bilayers and association with hydrophobic molecules. We are using OAR as a

possible ligand to identify protein translocating sites on the rough endoplasmic reticulum. The finding that OAR is immunologically distinct from native ovalbumin will enable us to identify which form is initially synthesized on ribosomes in the presence and absence of membranes.

The molecular biological aspects of our work are aimed at developing in vitro systems for membrane assembly in order to define structures in the membrane that allow recognition and translocation of secreted proteins and assembly of membrane proteins: 1) we are studying how to couple the in vitro synthesis of secreted protein such as immunoglobulin and ovalbumin from mRNA to membranes using rat liver microsomes as an acceptor membrane. 2) we are developping techniques to isolate and characterise ribosomal and mRNA of H. Halobium. In vitro translation systems are tested to translate H. Halobium message in general, and bacteriorhodopsin message in particular.

The studies of directed interaction between lipid vesicles and cells (Weinstein, Blumenthal) are further pursued. Those studies are applicable to problems in cell biology and to possible uses of liposomes in clinical therapy. The studies are aimed at developing ways to targeting drug-laden liposomes to particular cells in vitro, and to particular local areas, for example tumors and sites of infection, in vivo.

The development and application of mathematical techniques for describing receptor clustering on the plasma membrane continued (C. DeLisi). The results were incorporated into a theory of the response of sensitized basophils and mast cells which were proposed to explain, in terms of the physical chemistry of cell surface events, the biochemical pathway (histamine release, specific desensitization, non specific desensitization) selected by the cell. Aside from predicting the parameters of importance in the control of this choice, the theory also provides an explanation for the wide range of qualitative differences observed in dose response curves. The work is in collaboration with R. Siraganian, NIDR.

Experimental applications of the theory of cell surface events were extended to IgG complexes interacting with Fc receptors on macrophages (DeLisi, Dower, Segal). This work is relevant to immune complex effector mechanisms, and may also provide insight into the analysis of data on a number of other systems, such as those involving binding of hormones or neurotransmitters to cell surface receptors. J. Hiernaux and P. Baker (NIAID) continued their experimental work on immune response dynamics that was initiated as a consequence of our theoretical models of certain aspects of systemic regulation of the immune response. The model is intended primarily for T-independent antigens, but a number of regulatory features are expected to be general. Several predictions including oscillations in antibody affinity, are now being tested experimentally.

The work on assays has been extended to liquid column chromatography (DeLisi, Hethcote). We have developed a non equilibrium theory which includes diffusion, and have obtained analytic expressions relating elution profile characteristics to thermodynamic and kinetic parameters. The equations are currently being applied and tested by J. Inman (NIAID) and I. Chaiken (NIAMDD).

Another area of research within the laboratory concerns biological macromolecules and their properties. Stabilities of macromolecular conformations are determined by their interatomic interactions; the relative importance, for proteins, of various classes of interactions, short range and long range, is being assessed in detail(Jernigan, Miyazawa). Short and medium range interactions appear to determine most of the regular secondary structures such as α-helices and β-strands. Treatments using approximate energies of regular repeating conformations in proteins yield correct preferred conformations for about 2/3 of all residues. Several simple models of long range intramolecular interactions have been formulated to facilitate investigations of protein folding pathways. Availability of the new VAX 11/780 computer stimulated Monte Carlo generations of protein conformations and has lead to development of a method to statistically describe various unfolded stages for long range interaction models. From the calculated probabilities of each native long range contact, protein folding-unfolding pathways are constructed. Parallel pathways involving different parts of the molecule are observed, even for the very small bovine pancreatic trypsin inhibitor. These results have stimulated development of a simpler, more direct method for calculating characteristics of protein folding pathways.

More detailed calculations of conformational energies, requiring evaluation of all interatomic distances, are feasible for protein fragments. Such calculations have lead to postulation of two new binding sites for lysozyme (Pincus, Smith-Gill, Scheraga). These sites are being experimentally tested with monoclonal antibodies specific to the binding site. Similar calculations are being applied to the variable regions of the antigen combining site of myeloma immunoglobulins; these indicate substantial conformational variability for changed residues (Pincus, Potter, Feldmann). The leader peptide sequences, which are the portions of proteins involved in their transport through membrane, are being studied(Pincus, Klausner, Jernigan). They are a sequentially diverse class of peptides; however, their similar functions indicate that conformational similarities are probable.

Modeling of the endocrine systems has continued with emphasis on lipoproteins, the glucose-insulin system and receptors. Further extension of the model for lipoproteins has been made by studying direct triglycerides synthesis pathways for Low Density Lipoproteins (LDL) and High Density Lipoproteins (HDL), the kinetics of apoA and ApoE proteins cholesterol and modified LDL apoB (Wastney, Chu, Schwartz). Some of the kinetic studies have been extended to animals (monkeys and dogs) in collaboration with Drs. A. Scanu and P. Herbert.

The major effort in the glucose-insulin system involved the integration of the glucose and gluconeogenic precursors subsystems into a single, physiologically oriented model (Wastney). This has the advantage of sharing of information and constraints by the various subsystems and greatly tightens the overall model. In light of this it is now more meaningful to define and identify the various 'cycles' in glucose metabolism (Cori, alanine, glycerol, etc.). Studies of insulin secretion and its control have also been resumed (Covell).

Various receptor systems are being modeled. Specifically, the effects of secretogogues on clacium kinetics in the cell.

The modeling techniques have been further improved this year by implementing a new version of Conversational SAAM (CONSAM) on our VAX 11/780 computer. This new system has practically revolutionized our modeling and opened new possibilities for further extension and automation of the modeling process (Boston, Greif, Berman).

The modeling of special systems in collaboration with various investigators has continued.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08300-09 LMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

SAAM, Modeling and Applications

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mones Berman, Ph.D.	Chief	LMB NCI
	Ira Schwartz, Ph.D.	Staff Fellow	LMB NCI
	Martha Chu, Ph.D.	Visiting Fellow	LMB NCI
	David Covell, Ph.D.	Staff Fellow	LMB NCI
	Meryl Wastney, Ph.D.	Visiting Fellow	LMB NCI
	Raymond Boston, Ph.D.,	Dept. of Agriculture,	LaTrobe Univ., Australia
	Peter Greif, M.D.		
	Saul Kravitz		
	Sandor Joffee		LMB NCI
	Naomi Sager, Ph.D.	Linguistic String Lab.,	Courant Inst, N.Y. Univ.

COOPERATING UNITS (if any)

LaTrobe University, Australia; New York University, N.Y.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.5	PROFESSIONAL: 2.0	OTHER: .5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continuing development of a computer system (SAAM) for the simulation, analysis and modeling of bio-kinetic systems. Further development of a conversational mode of operation, increased versatility of applications and automation in modeling are in progress.

Analysis of various kinetic and metabolic systems by the use of mathematical models carried out with other investigators.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To develop a general purpose computer program for modeling biokinetic systems that may readily be used by investigators not sophisticated in mathematics or programming. Initiated in 1959, the program continues to be expanded and revised as new features are added.

Analysis of data on metabolic systems, and further development of modeling theory and techniques through the applications of the SAAM computer program.

Major Findings: Through the visits of Dr. Ray Boston from LaTrobe University, Australia, new features have been incorporated in conversational SAAM (CONSAM). These include the abilities to interfere with the least squares convergence procedures and to modify them to compensate for non linearities. The differential equations solution algorithms are also being expanded to take advantage of the latest state of the art in this area (Schwartz).

The SAAM programs are being applied to a variety of problems. A model was constructed for the kinetics of warfarin in dogs (Covell); HDL apo A kinetics were studied in the dog in collaboration with Dr. A. Scanu (Univ. of Chicago) (Chu), and in monkeys -- in collaboration with Dr. Peter Herbert (Unif. of Rhode Island) (Chu). Various other transport problems have also been modeled in collaboration with other investigators, or as part of a training activity.

A pilot study was initiated this year to develop data bases for various metabolic systems to serve as bases for the development and testing of models. The data bases will be contructed partly from available data and partly from literature data. Formatting of the literature data is now in progress in collaboration with Dr. Naomi Sager, who heads a computer linguistics group at New York University, NY.

Significance to Biomedical Research and the Program of the Institute: The methodology of modeling is most essential for studying the behaviour of systems for both normal and abnormal states. The SAAM computer program has been a most essential tool in the modeling of various systems described elsewhere in this laboratory report. Its wide use in other centers in the United States and elsewhere is further evidence of its value in biomedical research.

Proposed Course: This is a continuing process for the development of theory and for the application of modeling techniques.

Publications:

Boston, R., Greif, P., and Berman, M.: CONSAM-Conversational SAAM as a Modeling Tool. In Berman, M., Grundy, and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Boston, R.C., Greif, P.C., and Berman. M.: Conversational SAAM - an interactive program for kinetic analysis of biological systems. Computer Programs in Biomedicine. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08303-09 LMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Movement of Molecules in Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R.P. Blumenthal, Ph.D.	Chief, Membrane Structure and Function Section	LMB NCI
OTHERS:		
John N. Weinstein, M.D., Ph.D.	Investigator	LMB NCI
Paul R. Dragsten, Ph.D	Staff Fellow	LMB NCI
Richard D. Klausner, M.D.	Research Associate	LMB NCI

COOPERATING UNITS (if any)	P. Henkart, Ph.D.	IB NCI
	J. Handler, M.D.	LKEM NHLB
	M.C. Fishman, M.D.	LDN, NICHD

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Membrane Structure and Function Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

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1.5	1.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Movement of fluorescently labelled molecules (lipids, proteins, carbohydrates) on cell surfaces is observed by fluorescence microscopy, and quantitatively assessed by the technique of fluorescence photobleaching recovery (FPR). Lipids are usually free to move on the cell surface with diffusion constants of about 10^{-8} cm^2/sec. Movement of membrane proteins is often restricted by mechanisms, whose nature is unclear. Two systems were studied in which movement of cell surface components was restricted: 1) In epithelia it appears that the tight junction acts as a barrier to diffusion of certain plasma membrane components between apical and basolateral surfaces: membrane-bound lectins and some lipid probes are incapable of passing through the tight junction region of epithelial membranes. The lectins are immobilized on the cell surface, but the lipid probes diffuse freely in the membrane. The ability of a lipid probe to pass the tight junction is correlated with its ability to "flip-flop" to the inner monolayer of the cell membrane bilayer. 2) In neuroblastoma cells the vast majority of Con A binding sites, which exhibit lateral mobility in undifferentiated cells, become immobilized after induction of differentiation. The mechanism of this immobilization may involve linkage to the internal actin network.

Project Description:

Objectives: To study the role played in cell membranes by the mobility and distribution of cell surface receptors. To study the physiological significance of domains of lipid in membranes. To study factors which constrain the movement of membrane protein and lipids to specific areas of the cell surface.

Methods Employed: Diffusion of fluorescently labelled molecules (proteins, lipids, carbohydrates) on cell surface is measured by the technique of fluorescence photobleaching recovery (FPR): a small spot on the fluorescent-labeled surface is photolytically bleached by brief exposure to an intense laser beam and subsequent recovery of the fluorescence is monitored. Other techniques to measure movement of molecules in membranes are fluorescence polarization, fluorescence energy transfer, fluorescence life time henerogeneity analysis, and direction of shift of the phase transition temperature induced by probes incorporated into disaturated phosphatidylcholine vesicles.

Major Findings: 1. Membrane-bound lectins and some lipid probes are incapable of passing through the tight junction region of epithelial cell membranes. 2. The lectins are immobilized on the cell surface, but the lipid probes diffuse freely on both apical and basolateral surfaces. 3. Fluorescence quenching experiments on cells and on large unilammelar vesicles, labelled with lipid probes, indicate that the ability of a lipid probe to pass the tight junction is correlated with its ability to "flip-flop" to the inner monolayer of the cell membrane bilayer. 4. In undifferentiated neuroblastoma cells Con A binding sites exhibit lateral mobility comparable with that of surface glycoproteins of other cells; after induction of differentiation, the vast majority of Con A binding sites become immobilized. 5. Cytochalasin B reduced the percentage of immobile sites in differentiated cells from 79 to 23%, and the distribution of internal actin, as revealed by the fluorescent ligand for actin, NBD-phallacidin, correlated with that of Con A receptors; those data suggest that the mechanism of immobilization may involve linkage to the internal actin network.

Significance for Biomedical Research and the Program of the Institute: Mobility and distribution of cell surface components are considered to have important implications for cell transformation and for many aspects of the physiology of normal and tumor cells.

Proposed Course: A. Epithelial cells: Pursue the following questions: 1. The control of movement of lectin binding sites by the cytoskeleton (redistribution after disrupting the monolayer does not involve free diffusion). 2. The nature of the selectivity filter for lipid probes at the tight junction. 3. Possible differences in lipid composition between apical and basolateral membranes. 4. The development of membrane asymmetry in isolated cells growing in culture. B. Domains: Investigate the possible physiological significance of lipid domains in membranes (e.g. for biosynthetic incorporation of proteins into membranes). C. Cytotoxic T-cells: Investigate the role of cell surface receptor mobility in dual recognition and the cytotoxic response.

Publications:

Fishman, M.C., Dragsten, P.R., and Spector, I.: Immobilization of concanavalin A receptors during differentiation of neurobluotoma cells. Nature (Lond.) 290: 781-783, 1981.

Maxfield, F.R., Willingham, M.C., Pastan, I., Dragsten, P.R., and Cheng, S.-Y.: Binding and mobility of the cell surface receptors for 3,3',5-triiodo-L-thyronine. Science. 211: 63-65, 1981.

Lucas, D.L., Dragsten, P.R., Robertson, D.M., and Bowles, C.A.: Increased lateral diffusion of a lipid probe is evident in the plasma membranes of elicited macrophages. J. Reticul Soc. In press.

Maxfield, F.R., Willingham, M.C., Haigler, H.T., Dragsten, P.R., and Pastan, I.H.: Binding, surface mobility, internalization and degradation of rhodamine-labelled α-2-macroglobulin. Biochemistry. In press.

Klausner, R.D., and Wolf, D.E.: Selectivity of fluorescent lipid analogues for lipid domains. Biochemistry. 19: 6199-6203, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08306-09 LMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Kinetic Modeling of Human Plasma Lipoprotein Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mones Berman, Ph.D.	Chief	LMB NCI
OTHERS:	Meryl Wastney, Ph.D.	Visiting Fellow	LMB NCI
	Martha Chu, Ph.D.	Visiting Fellow	LMB NCI
	Scott Grundy, M.D.	Veterans Administration, San Diego, CA	
	Waldo Fisher, M.D.	Univ. of Florida, Gainsville, FL	
	Charles Schwartz, M.D.	Veterans Administration, Richmond, VA	
	Claude Malmendier, M.D.	Univ. of Brussels, Belgium	

COOPERATING UNITS (if any)

VA Hospital, Richmond VA; VA Hospital, San Diego, CA; UCSD School of Medicine, Univ, of Fla.; Univ, Brussels

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NCI, NIH, Bethesda, Maryland 20205

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1.3	1.3	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Kinetic models of plasma apoproteins, cholesterol and triglyceride are being constructed based on data from experiments in man. The models are used to integrate plasma lipoprotein interactions with enzymes and receptors and to provide a better understanding of plasma lipoprotein synthesis and metabolism in health and disease. The models are particularly useful for the rigorous testing of hypotheses, the design of experiments, and the quantification of the effects of various perturbations.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To develop a qualitative and quantitative understanding of lipoproteins metabolism in man and to identify abnormalities and drug effects through the modeling of apoprotein, cholesterol, and triglyceride kinetics. Because of the need for diverse extensive data on a variety of patients, collaboration with several experimental groups is maintained.

Methods Employed: Mathematical modeling is the tool used for the integration and analysis of the data. This is performed with the help of the SAAM computer modeling program.

Major Findings: The modeling continues along various routes of the lipoprotein system. The kinetics of asialated and sialated LDL were examined (Wastney) and the kinetics of apoE in normal and type III patients were modeled (Chu). Although no definitive conclusions could be drawn from these kinetics, several interesting conjectures were advanced. In the case of apo E kinetics - it seems possible that the tracer data permit the identification of an E-III component in normal apoE and a change in affinity for apoE in type III patients.

The overall model for triglycerides (TG) kinetics (Zech, et al.) was further extended to include TG exchanges between HDL and VLDL and the incorporation of TG exchanges between HDL and VLDL and the incorporation of TG in nearly synthesized IDL and LDL (Berman). The kinetics of cholesterol ester (Schwartz) were studied and the rates of exchange between the various plasma components determined. It appears that free cholesterol of HDL is the primary substrate for newly formed cholesterol ester.

A book on lipoprotein kinetics and modeling was edited and is now in the process of publication (Academic Press).

Significance to Biomedical Research and the Program of the Institute: Modeling is important to identify the abnormalities in metabolism responsible in hyperlipemics, diabetics, and other metabolic disorders, given a very complicated system with multiple interactions. It is hoped that by identifying the mechanisms responsible for abnormalities in lipid metabolism and their relations with intermediate metabolism, appropriate treatment can be more specifically geared to abnormalities. Clinically this is relevant to atherosclerosis and disorders associated with athersclorsis such as cardiovascular disease, diabetes, cachexia, and other metabolic disorders involving lipids.

Publications:

Fisher, W.R., Zech, L.A., Bardalaye, G.W. and Berman, M.: The metabolism of apolipoprotein B in subjects with hypertriglyceridemia and polydisperse LDL. J. Lipid Res. 21: 760-774, 1980.

Foster, D.M., and Berman, M.: Hydrolysis of rat chylomicron acylglycerols: a kinetic model. J. Lipid Res. 22: 506-513, 1981.

Berman, M.: ApoC Kinetics. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Schwartz, C.C., Berman, M., Halloran, L.G., Swell, L. and Vlhacevic, Z.R.: Determination of Total Body Disposal in Man: Special Role of HDL Free Cholesterol. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Wastney, M.E., Riemke, R., Malmendier, C.L., and Berman, M.: Heterogeneity of Low Density Lipoprotein: Kinetic Analyses of Asialated Lipoproteins. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Berman, M., Beltz, W.F., Riemke, R., and Grundy, S.M.: VLDL-RG Exchange with HDL-TG In Vivo. In Berman, M., Grundy, S.M., and B.V. Howard (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Grundy, S.M., Mok, H.Y.I., Zech, L., and Berman, M.: Influence of nicotinic acid on metabolism of cholesterol and triglycerides in man. J. Lipid Research, 21: 760-774, 1980.

Le, N-A., Grundy, S.M., and Berman, M.: A Reduced Model for Very Low Density Lipoprotein Triglyceride Metabolism. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Berman, M., and Chu, M.: A Possible Interpretation of ApoE Kinetics in Man. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Berman, M.: Kinetic Analysis and Modeling: Theory and Applications to Lipoproteins. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08320-06 LMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Macromolecular Conformations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert L. Jernigan, Ph.D.	Theoretical Physical Chemist	LMB NCI
OTHERS:	Sanzo Miyazawa, Ph.D.	Visiting Fellow	LMB NCI
	Matthew Pincus, M.D., Ph.D.	Expert	LMB NCI

COOPERATING UNITS (if any)

Richard Feldmann, CCB, DCRT, NIH
Michael Potter, LCBGY, NCI, NIH

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NCI, NIH, Bethesda, Maryland, 20205

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2.0	2.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Protein conformations have been investigated by means of several types of calculations. These include: 1) calculation of approximate energies for regular secondary regions, based on electrostatic energies, 2) investigations of folding pathways by extensive Monte Carlo generation of peptide conformations, in which the native conformation is favored but atomic overlaps are not permitted, and 3) more detailed energy calculations on protein fragments. In the first case, it was determined that, similar to other secondary conformation prediction methods, only about 2/3 of the residues are favored in their native conformation, if only local and medium range interactions are included. The implication is that long range interactions modify conformations of significant numbers of residues. Definite folding pathways were obtained for pancreatic trypsin inhibitor from the random generation. The activated state for the folding is found to correspond to the formation of the two strand beta sheet. Subsequently, multiple folding pathways were obtained. In the third case above, hypervariable regions of myeloma immunoglobulins have been optimized within the constraints imposed by the remainder of the molecule.

Project Description:

Objectives: To develop theoretical methods adequate to determine the most significant macromolecular conformations. The principal molecules of interest are proteins. At the most elementary level, we wish to predict protein conformations from the sequences and to determine the relative importances of various intramolecular interactions. However, methods are limited and cannot yet be expected to yield complete three dimensional conformations of large molecules; presently feasible calculations are: 1) approximate energy calculations of entire molecules or 2) more detailed energy calculations for fragments of large proteins. In the former case, we would like to be able to randomly generate conformations and use calculated conformational probabilities to construct folding pathways. In the latter case, we would like to be able to determine a protein's conformation when changes in sequence are restricted to local regions, by using a homologous known structure as the basis for a structure. An ultimate goal is to understand effects of changes in peptide sequences on biological function.

Methods Employed: Approximate methods for calculating conformational energies, based substantially on electrostatic energies, have been developed. These were utilized with methods for selecting best sets of regular secondary conformational regions. Methods of selection included minimizing the energy, and two alternative methods derived by dynamic programming concepts.

Monte Carlo methods for generating total molecular conformations, in which atomic overlaps are prohibited, were utilized in treating folding pathways. Molecular conformations were generated for all regions of energy; this permits a detailed consideration of folding pathways, by sampling molecules which are denatured to different extents. The folding-unfolding process of reduced bovine pancreatic trypsin inhibitor was investigated with an idealized model employing approximate free energies. Intra-residue energies consist of two terms, an empirical part taken from the observed frequency distributions of (ϕ ,ψ) and an additional favorable energy assigned to the native conformation of each residue. Inter-residue interactions were simplified by assuming that there is an attractive energy operative only between residue pairs which are in close contact in the native structure. More than 200,000 molecular conformations, ranging from the native state to the denatured state, were randomly generated by changing the sampling bias. Each conformation was classified according to its conformational energy, E; a conformational entropy, S(E) was estimated for each value of E from the number of samples. Inter-residue contact probabilities, at different stages of folding, serve to characterize in detail the folding process. From such results, probable pathways for the folding-unfolding transition can be constructed, by assuming small conformational changes between intermediates.

The detailed energy calculations include interactions between all interatomic pairs; parameters in these calculations were originally derived from experimental crystal data. A large number of starting conformations were chosen and the conformations were varied while seeking energy minima. A number of the invariant residues on each side of the variable region were included in the fragment whose conformation is investigated.

Major Findings: The present secondary structure calculations yield results that are correct for about 2/3 of the residues in a protein. This is, however, not sufficient for making detailed statements about overall three dimensional protein conformations. In the simplified secondary energy calculations, the best results indicate that intramolecular conditions vary significantly, in the sense of a mean field, from one protein to another; this result, together with a number of points of flexibility, which are sites locally indifferent to conformation, may provide substantial explanation of the errors manifested in secondary structure predictions.

The dependence of the entropy S(E) on energy reveals that the folding-unfolding transition for this idealized model of pancreatic trypsin inhibitor is an "all-or-none" type; such behavior can be attributed to the highly specific long range interactions. The details of the folding pathways obtained for pancreatic trypsin inhibitor correspond to four strands folding: 1) At a preliminary stage, folding begins with appearance of nuclei at a beta turn and at the alpha helix. 2) These grow to include the native pair of hydrogen bonded beta strands. This two strand nucleus includes intact regular secondary conformations, as well as the inter-strand sheet contacts, and corresponds to an activated state with the highest free energy on the pathway. 3) Additional native long range contacts are completely formed between this nucleus and either the amino terminus or the carboxyl terminus strand, yielding a mixture of two different three strand products. 4) In a final step, the remaining free strand is folded. Steps 3 and 4 correspond to multiple folding pathways. The method for calculating statistical folding pathways is general and could be applied to any set of calculated conformational energies.

The conformation of the antigen combining site for the myeloma protein MOPC539 has been calculated, with most sequentially invariant residues fixed in accordance with the X-ray structure of the immunoglobulin MOPC603. A plausible structure of low energy has been obtained; invariant residues have been determined to be virtually unaffected by the changes in sequence between the two molecules. The conformations of the variable regions of MOPC539 differ significantly from those of the corresponding variable regions of the "parent" protein MOPC603, particularly in regions in which insertions and deletions occur. These differences in conformation are being investigated to determine how they affect specificity of interaction with various antigens.

Significance to Biomedical research and the Program of the Institute: Developing theoretical methods to comprehend the origins of protein conformations would permit a better understanding of protein structure and function.

Proposed Course: Effects of side chain conformations on calculated secondary region energies will be investigated. Previous methods preaveraged side chain conformations; this had the effects of significantly weakening interactions involving dipolar side chains.

More detailed evaluations of the effects on the pathways of excluded volume and the strengths of intramolecular interaction energies will be pursued. This is feasible by means of a new matrix method for determining folding pathways. Application of these methods to other proteins will follow. Applications of theories of phase transitions based on rodlike molecules are anticipated in modeling polypeptide transitions to ordered, high density forms such as beta

sheets. In order to comprehend the appearance of favorable long range interactions, probabilistic studies of the effects of specific classes of interactions on ring closure are anticipated.

Also planned are calculations on the class of small peptide fragments termed "leaders" which are involved in transport of proteins through membranes. Secondary conformation calculations may serve as an initial guide in choosing starting conformations for the necessary more detailed energy calculations.

Publications:

Jernigan, R. L., Miyazawa, S., and Szu. S. C.: Stabilization of regular conformational regions in proteins by intraregion electrostatic interactions. Macromolecules, 13: 518-525, 1980.

Jernigan, R. L., Miyazawa, S., and Szu, S. C.: Electrostatic interactions and secondary structure in proteins. Biophysical J. 32: 93-95, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08323-06 LTB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Assay Quantitation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Charles DeLisi, Ph.D. Senior Investigator LMB NCI

OTHERS: Herbert Hethcote, Ph.D. Mathematician, IPA LMB NCI

COOPERATING UNITS (if any) John Inman Ph.D., Laboratory of Immunology, NIAID; Irwin Chaiken, Ph.D., Laboratory of Chemical Biology, NIAMD; Prof. Pierre Masson, Inst. Cellular Molec. Pathol., Brussels; Prof. Gregory Siskind, Div. of Immunol., Cornell Univ. Med. School.

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NCI, NIH, Bethesda, Maryland 20205

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0.7	0.7	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) A theory has been developed to describe a heterogeneous, non equilibrium system in which diffusion, transport and chemical reaction are all occurring simultaneously. The variables entering the equations are: bead bound antigens, mobile antibodies that react specifically with the antigens but which have a distribution of rate and equilibrium constants for them, and mobile antigens which inhibit the antibody-bead bound antigen interaction. Characteristics of the affinity and rate constant distributions are related to characteristics of the elution profile by relatively simple expressions even for systems in which neither chemical equilibrium nor a local steady state has been established. The effect of movement in and out of the bead under non ideal conditions (activity coefficient different from unity) is included. An important aspect of the development is the theoretical relation between the affinity of antibody for a surface bound antigen as opposed to the affinity for a free antigen. The theory suggests a method for obtaining the affinity constants for both reactions. Extensions and applications of a mathematical model of the hemolytic plaque assay continued. In addition, work was begun on the development of equations to be used in the quantitation of nonisotopic agglutination assays, which can be used as substitutes to radio Immunoassay.

Project Description:

Objectives: To develop a simple, fast, widely available method for obtaining quantitative physical chemical information for complex reaction systems. To develop quantitative methods for obtaining kinetic and thermodynamic information on antigen antibody reactions at a single cell level. To develop methods for increasing assay reliability and precision.

Methods employed: Mathematical models; mathematical analyses of data.

Major findings: The chromatography theory is still in its early stages of development, but the equations derived have been applied by Dr. John Inman, NIAID to determine the equilibrium constant for an anti TNP Myeloma. He obtains a value within 5% of the accepted value that had previously been determined by dialysis. Work on applications of the plaque assay, especially as a method for analysis of anti-idotypic antibodies, continued but at a slow pace. Most effort was directed toward developing a quantitative theory of non isotopic aggregation assays whose use as replacements for radio immunassays is continuing to increase. Equations were derived that will allow assay optimization including an analysis of error structure of the system.

Significance to Biomedical Research and the Program of the Institute: The chromatography project is the basic component of projects related to the physical chemistry of cellular recognition and regulation. It will provide the thermodynamic and kinetic data required to develop a quantitative understanding of cellular regulation. The work on plaques is intimately related to the project on B cell regulation. It makes possible a method for studying cellular selection. It also provides a potentially new and valuable method for quantitating under appropriate conditions, the antiidiotypic antibody response. Immunoassays are important, for among other things, the detection and quantitation of low concentrations of ligands in the serum. Aside from the ability to detect abnormally low or high values of hormones that are normally present, they are potentially useful in the detection of tumor associated antigens.

Proposed Course: The reliability of column chromatography for obtaining various types of physical chemical information depends in a complicated way on the interplay between transport velocity, various types of diffusion phenomona, and movement in and out of bead, as well as on properties intrinsic to the reaction being studied. We will develop procedures for optimizing column parameters for the determination of rate constants, and information on heterogeneity in equilibrium constants. We will develop an understanding of the theoretical limits of the methods, and the techniques will be implemented experimentally by Drs. Inman and Chaiken for different systems. Simulation of the system in the absence and presence of errors in order to develop methods for optimization under a variety of experimental conditions. The mathematical analysis of augmentable plaques as a quantitative method for monitoring the dynamics of suppressive complexes in the immune response, will be implemented.

Publications

DeLisi, C. and Hethcote, H.: A theory of column chromatography for sequential reactions in heterogeneous non equilibrium systems: Application to antigen antibody reactions. Analytical Chemistry Symposia Series. Amsterdam, Elsevier. In press.

DeLisi, C. and Hiernaux, J.: Mathematical analysis of augmentable plaque forming cells: a quantitative method for monitoring auto antiideotypic antibody. In DeLisi, C., and Hiernaux, J. (Eds.) Regulatory Implications of Oscillatory Dynamics in the Immune Response. Florida, CRC Press, 1981. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08331-05 LMB

PERIOD COVERED: October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Glucose-Insulin Kinetics. Modeling of Carbohydrate Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mones Berman, Ph.D.	Chief	LMB NCI
OTHERS:	Meryl Wastney, Ph.D.	Visiting Fellow	LMB NCI
	David Covell, Ph.D.	Staff Fellow	LMB NCI
	Susan E.H. Hall, Ph.D.	Ottawa Civic Hospital, Canada	
	Jesse Roth, M.D.	Chief	DB NIAMDD
	Reubin Andres, M.D.	Gerontology Research Center, Baltimore	
	Jordan Tobin, M.D.	Gerontology Research Center, Baltimore	

COOPERATING UNITS (if any)

Clinical Physiology Branch, GRC, NIA, Baltimore; Diabetes Branch, NIAMDD; Dept. of Physiology, Univ. of Ottawa; Div. of Metabolism, Ottawa Civic Hospital, Ontario

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Kinetic models of the glucose regulatory systems are being developed for man and other animals in collaboration with several experimental groups. These include models of the distribution, metabolism and receptor binding of insulin, using data from both radioiodinated and native insulins, and the production and utilization of glucose as elucidated by tracer studies with labeled glucose, alanine and lactate. Models of the role of insulin in the control of glucose utilization and production are also being studied. The kinetics of ketones in man have also been studied and modelled.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To develop a qualitative and quantitative understanding of glucose homeostasis including the role which various hormones play in the production and utilization of glucose and the role which glucose plays in the secretion of insulin in normal and perturbed metabolic states. To study the various precursors of glucose and other substrates that relate to glucose utilization.

Major Findings: The development of a glucose-insulin model is a long range process of integration of various subsystems. In the present phase the kinetics of the various glucose precursors (alamine lacatate, pyruvate, etc) have been integrated into a general model with glucose to permit further development and testing of the total model and its parts. Data from the literature were imposed as additional constraints on the model resulting in a more fully defined and compatible model (Wastney). Newer studies on insulin secretion obtained from the NIA group (Drs. Andres, Tobin) have been examined with our previously developed model for insulin secretion, and additional improvements in the model were incorporated (Covell). The total glucose-insulin feedback loop was examined for its potential use as a diagnostic and therapeutic tool (Covell).

An additional component in the study of intermediate metabolism was introduced by modeling the kinetics of ketone bodies in man (Wastney, Hall). Some anamolies have been identified in the analysis of ketone kinetics using conventional methods (areas under specific activity curves, etc), and a model is being developed to deal with these. It is proposed that either there is a labeled moiety other than aceto-acetate and beta-hydroxybutanata in plasma to account for the tracer and tracee data or else there are experimental artifacts that need to be resolved.

Significance to Biomedical Research and the Program of the Institute: Carefully tested models provide predictions as to how a complicated regulatory system will respond to a given perturbation. The clinically or experimentally observed responses seen in various altered metabolic states can thus be analyzed using such models to gain insight into the basic perturbations which have occurred in these states. Obesity, diabetes, aging and the cachexia of advanced cancer are examples of metabolic conditions in which glucose homeostasis is perturbed and can thus be better understood by use of these models.

Proposed Course: 1) To continue the study of the regulatory behavior of the glucose-insulin system in an attempt to identify the primary perturbations in the obese, diabetic and aged subjects for which data are available. 2) To extend the insulin model to account for differences between studies using radioiodinated and native insulin, and to include new information on the binding and degradation of insulin. 3) To study the biological effects of insulin action. 4) To identify the physiological process reflected in the models.

Publications:

Foster, D.M., Hetenyi, G. Jr., and Berman, M.: A model for carbon kinetics among plasma alanine, lactate and glucose. Amer. J. Phys. 239: E30-E38, 1980.

Hetenyi, G. Jr., Layberry, R.A., Foster, D.M., and Berman, M.: Transfer of carbon atoms among circulating glucose, alanine, and lactate in pancreatectomized dogs. Amer. J. Phys. 239: E39-E44, 1980.

Lowry, S.F., Foster, D.M., Norton, J.A., Berman, M., and Brennan, M.F.: Glucose disposal and gluconeogenesis from alanine in tumor-bearing Fischer 344 rats. J. Nat. Cancer Inst. 66: 653-658, 1981.

Goebel, R., Berman, M., and Foster, D.: A mathematical model for the distribution of isotopic carbon atoms through the tricarboxylic acid cycle. Fed. Proc. In press.

Hall, S.E.H., Bratten, J.T. McKendry, J.T.R., Bolton, T., Foster, D. and Berman, M.: Normal Alanine: glucose relationships and their changes in diabetic patients before and after insulin treatment. Diabetes. 28: 737-745, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08335-05 LMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
"Targeting" Liposomes for Selective Interaction with Specific Cells and Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John N. Weinstein, M.D., Ph.D.	Investigator	LMB NCI
OTHERS:	Robert P. Blumenthal, Ph.D.	Chief, Membrane Structure and Function Section	LMB NCI
	Evelyn Ralston, Ph.D.	Visiting Associate	LMB NCI

COOPERATING UNITS (if any)
S.O. Sharrow, J. Wunderlich, W. Terry, IB, NCI;
M. Yatvin, Univ. of Wisconsin; L. Leserman, Centre d'Immunologie, France;
R. Magin, D. Zaharko, R. Cyzyk, LCP, NCI.

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Membrane Structure and Function Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER: 0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

When hapten-modified liposomes were "targeted" to myeloma cells or to human lymphocytes, they bound in large numbers to the cell membranes, but their contents were not internalized. However, when liposomes opsonized with IgG were presented to murine P388D$_1$ cells, they were readily endocytosed. Uptake was specifically mediated by the Fc receptor on the cell. If methotrexate (MTX) was encapsulated in the liposomes, it escaped from the phagolysosomal apparatus to reach a cytoplasmic target and affect the physiology of the cell. We have developed a method for covalently attaching antibody and other ligands to liposomes.

We have designed "temperature-sensitive" liposomes that release an entrapped drug locally at temperatures obtainable by mild hyperthermia, for example in the treatment of tumors. In the presence of serum the ratio of drug release at 43° to that at 37° can be made greater than 100:1. We find that (i) such liposomes deliver at least 14 times as much MTX to heated murine tumors as to unheated control tumors, (ii) the drug reaches its target enzyme in the tumor cell cytoplasm, and (iii) tumor growth can thus be delayed. With large unilamellar liposomes, serum leads to even faster and more useful release at T_c.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To investigate the use of liposomes both in cell biology and in clinical therapy. Within this broad context,

(1) To explore the use of antigen-antibody interactions to achieve selective association of liposomes with particular cell types;

(2) To develop synergistic interactions between "temperature-sensitive" liposomal drug carriers and hyperthermic treatment;

(3) To identify the mechanisms of spontaneous, serum-induced, and osmotically-induced release of solutes from liposomes.

Non-standard Methods Employed: (a) Preparation of liposomes, by bath and probe sonication. (b) Investigation of liposome-cell interactions using the fluorescence-activated cell sorter. (c) Dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory for the purpose. (d) Measurements of release of solute from liposomes by "fluorescence self-quenching". (e) Determination of the mobility of vesicles bound to cells, using fluorescencephotobleaching. (f) Microwave heating of tumors.

Major Findings:

Objective 1: (a) Bivalent antibody selectively binds DNP-bearing liposomes to TNP-bearing lymphocytes, but the binding does not increase delivery of liposome contents to the cell interior (beyond the amount internalized spontaneously). (b) Endogenous surface IgA on cells of the murine myeloma MOPC 315 can bind liposomes bearing the appropriate hapten (DNP) to the cell surface. However, as in the lymphocyte system, binding does not increase delivery to the cytoplasm. (c) Lipid vesicles containing fluorescent molecules are potentially useful as markers for sparse or low-affinity cell-surface determinants. They can be made to contain many fluorophore molecules, thus amplifying the signal. They give very low non-specific background since the fluorophore is sequestered, and appropriate lipids are not very "sticky". (d) Liposomes are removed from the circulation of a mouse much faster if the mouse carries a myeloma secreting antibody to a hapten on the liposome. (e) Antibody-mediated binding of methotrexate-containing vesicles to MOPC 315 and TEPC 15 myeloma cells did not lead to entry of drug into the cells and inhibition of their metabolism. (f) IgG opsonized DNP-vesicles are bound in large numbers to F_c receptor-bearing cells (murine $P388D_1$), and are then endocytosed. Liposome-encapsulated MTX then escapes the phagolysosomal system to reach a cytoplasmic target (dihydrofolate reductase) and affect the physiology of the cell. (g) IgG, protein A, avidin, and other ligands can be coupled efficiently to liposomes by use of the heterobifunctional cross-linking agent N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate. This method of coupling results in only minimal aggregation and little leakage of vesicle contents. Liposomes bearing covalently coupled mouse monoclonal antibody against human β_2-microglobulin bind specifically to human cells, but not to mouse cells.

Objective 2: (a) Small unilamellar vesicles of 3:1 dipalmitoylphosphatidylcholine - distearoylphospatidylcholine release their contents very slowly at 37°C, much faster at 41 - 46°C. Such "heat-labile" liposomes appear useful in achieving high drug concentrations selectively in local areas of hyperthermic treatment, for example, in the treatment of tumors. (b) The temperature-dependence of the release

can be enhanced by increasing the rate of temperature change, by using multilamellar vesicles in place of the unilamellar ones, and most markedly, by the presence of serum in the medium. Ratios of greater than 100:1 can be obtained for release (of a fluorescent marker) at 43 and at 37°. (c) The effect of serum is largely due to interaction of serum lipoproteins (VLDL, IDL, LDL, and HDL) with the liposomes. (d) Four times as much methotrexate was delivered to subcutaneous Lewis lung tumors heated to 42° as to unheated controls in the same animals at 36°; with L1210 tumor the ratio was 14:1. (e) Growth of the L1210 tumors was delayed by such treatment more than could be accounted for by the separate effects of heating and liposomal drug administered separately. (f) Large unilamellar vesicles are stable below T_c but release their contents within a few seconds upon passage through T_c with serum.

Objective 3: (a) Release of carboxyfluorescein from small unilamellar vesicles takes place by "leakage", not by an all-or-nothing "rupture" of the vesicle. (b) The rate constant for leakage increases in inverse proportion to the hydrogen ion concentration of the medium. (c) Liposomes of dioleoyl lecithin leak their contents and form structures with a characteristic appearance in negative-staining electron microscopy when allowed to interact with HDL or LDL. The interaction is faster and more pronounced with isolated HDL apolipoprotein than with the whole lipoprotein particle. (d) Liposomes bearing the DNP-hapten can be made to release carboxyfluorescein in the presence of complement and IgG anti-TNP. Fluorescence self-quenching provides the most sensitive technique available for continuously monitoring such processes. (e) Cholesterol-containing liposomes can pass intact, and without releasing much of their contents, from the peritoneum to the blood-stream of a mouse. This finding is potentially important for possible clinical instillations of liposomes I.P.

Significance to Biomedical Research and the Program of the Institute: The three objectives listed clearly relate to the possibilities for using liposomes in tumor therapy. A major barrier to such efforts has been the difficulty of directing liposomes to particular cells or anatomical sites. The studies of antibody-mediated "targeting" suggest a way to achieve selectivity but also demonstrate an additional problem: how to get the liposome and its contents into the cell after binding. The studies of synergism between liposomes and hyperthermia indicate a promising new way to achieve selective delivery. Use of the liposome as a hapten-carrier for analysis and sorting of cell populations may be useful in a number of areas of cellular and tumor immunology. The covalent attachment of immunoglobulin and ligands to liposomes will make possible a wide range of cell biological studies.

Proposed Course: Each of the objectives specified earlier will be pursued. In the case of the hyperthermic system, emphasis has now shifted from in vitro to in vivo approaches and also to theoretical analysis of the pharmacokinetics. Studies of antibody-mediated "targeting" have now been extended to include assays of the physiological effects of delivered drugs in vitro, as well as the distribution of fluorescence markers.

Publications:

Ralston, E., Blumenthal, R., Weinstein, J.N., Sharrow, S.O., and Henkart, P.: Lysophosphatidylcholine in liposomal membranes: Enhanced permeability but little effect on transfer of a water-soluble fluorescent marker into human lymphocytes. Biochim. Biophys. Acta. 597: 543-551, 1980.

Chused, T.M., Sharrow, S.O., Weinstein, J.N., Ferguson, W.J., and Sternfeld., M.: XRITC; A new dye for two-color immunofluorescence. J. Histochem. and Cytology. In press.

Leserman, L.D., and Weinstein, J.N.: Receptor mediated binding and endocytosis of drug-containing liposomes by tumor cells. In Tom, B.H. and Six, H.R. (Eds.): Liposomes and Immunobiology. Amsterdam, Elsevier, 1980, pp. 241-251.

Leserman, L.D., Weinstein, J.N., Moore, J.J., and Terry, W.D.: Specific interaction of myeloma tumor cells with hapten-bearing liposomes containing methotrexate and carboxyfluorescein. Cancer Research. In press.

Leserman, L.D., Weinstein, J.N., Blumenthal, R. and Terry, W.D.: Receptor-mediated endocytosis of antibody-opsonized liposomes by tumor cells. Proc. Natl. Acad. Sci. 77: 4089-4093, 1980.

Magin, R.L., and Weinstein, J.N.: Selective delivery of drugs in "temperature-sensitive" liposomes. In Tom, B.H. and Six, H.R. (Eds.): Liposomes and Immunobiology. Amsterdam, Elsevier, 1980, pp 315-325.

Leserman, L.D., Barbet. J., Kourilsky, F.M., and Weinstein, J.N.: Liposomes directed to specific cellular targets by covalently-coupled monoclonal antibody, protein A, and avidin. Nature. 288: 602-604, 1980.

Weinstein, J.N., Klausner, R.D., Innerarity, T.L., Ralston, E., and Blumenthal, R.: "Phase transition release" (PTR), a new approach to the interaction of proteins with lipid vesicles: application to lipoproteins. Biochim. Biophys. Acta. In press.

Pitas, R.E., Innerarity, T.L., Weinstein, J.N., and Mahley, R.W.: Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. Atherosclerosis. In press.

Yatvin, M.B., Muhlensiepen, H., Porschen, W., Weinstein, J.N., and Feinendegen, L.E.: Selective delivery of liposome encapsulated cis-dichlorodiammineplatinum (II) by heat: Influence on tumor drug uptake and growth. Cancer Research. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08337-03 LMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Complement Fixation and Activation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Charles DeLisi, Ph.D. Senior Investigator LMB NCI

COOPERATING UNITS (if any)
Dr. Michael Boyle and Dr. Tibor Borsos, Laboratory of Immunobiology, DCBD, NCI; Prof. Frederik Wiegel, Dept. of Physics, Twente Univ. of Tech., Enschede, Netherlands

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The development of mathematical theories for various components of the classical complement pathway, and their application to data analysis was continued. In particular mathematical methods were developed and applied to the analysis of the C9 attack sequence and C1Q antibody recognition. A new theory for the recognition of IgG on a fluid surface by C1Q was also developed.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To obtain information about (1) the final stages of lysis, including distribution of hole sizes; (2) the effect of epitope density on lysis; (3) the effect of Ig concentration and cell surface distribution on the kinetics of lysis.

Major findings: Data fits of the theory predict that C9 binding is negatively cooperative. The result suggests the possibility of a conformational change in EAC1-8. Recent independent experimental studies corroborate this prediction. Data analysis also indicates clustering of the EAC1-9 complexes. The equilibrium constant for C9 binding at low concentrations was also obtained from the best fit of the data. The value of $5 \times 10^{11}\ M^{-1}$ is within experimental error of numbers obtained using labelled C9. The agreement indicates the possibility of using the relation between (C9) dose and response (hemolysis) as a method for obtaining affinities without using labelled ligand.

A theory of fluidity effects for C1Q fixation by IgG predicts an optimum fluidity for fixation. At fluidities higher than the optimum (protein diffusion coef. $\geq 10^{-10} cm^2/sec.$) a precipitous drop in the fixation probability occurs. This effect may provide the cell with switching mechanism whereby relatively minor changes in membrane components can have a pronounced effect on IgG mediated lysis.

Significance to Biomedical Research and the Program of the Institute: Complement is important in host defense systems. Moreover, the experimental systems are an excellent model for understanding initial stages of biological events and correlating molecular and cellular events. The work interfaces with other projects on cell surface events, effector functions and cellular activation.

Proposed Course: Application to data, followed by additional experiments as indicated by the analysis. Development of mathematical methods for analyzing other steps in the classical complement pathway as time and support permit. Testing prediction of the new theory if fluidity effects.

Publications:

DeLisi, C., Boyle, M., and Borsos, T.: Mathematical analysis of the reaction of EAC1-8 with C9. Identification of parameters defining conditions for molecular titration. J. Immunol. 125: 2334-2338, 1980.

DeLisi, C., Boyle, M., and Borsos, T.: Analysis of the colloid osmotic step of complement mediated immune hemolysis. J. Immunol. 125: 2055-2062, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08340-03 LMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Physical Chemistry of Antibody Effector Functions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Charles DeLisi, Ph.D. Senior Investigator LMB NCI

COOPERATING UNITS (if any) Dr. Ruben Siraganian, Clinical Immunology Section, NIDR; Prof. George Barisas, Biochemistry Dept. Univ. of St. Louis Med. School; Alan Perelson, Ph.D., Los Alamos National Lab., Los Alamos, NM; Dr. David Segal, Immunology Branch, DCBD, NCI; Dr. Steve Dower, Immunology Branch, DCBD, NCI

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Work on the basophil system was continued with the development of a new theory of the kinetics of activation and specific desensitization of cells from allergic and immunized individuals. The theory has been applied to the analysis of a wide variety of data. We also developed new methods, based on measurements of the kientics of cell activation, for determining whether or not the descending limb of biphasic dose response curve falls because of insufficient cross-linking. We studied equilibrium and kinetic properties of IgG diagomers of defined size interacting with Fc receptors on a macrophage-like cell line. The results of the equilibrium studies provided the first experimental evidence in support of our predictions that receptor cross-linking can lead to non linear Scatchard plots. In addition, the data suggested two different types of binding sites for dimeric and trimeric oligomers, but only a single type for monomers. The maximum affinity enhancements -- 200 for dimeric relative to monomeric and 2.5 for trimeric relative to dimeric, indicate considerable strain or large differences in the entropic parts of the equilibrium constants for solution phase as apposed to cell surface reactions. The dissociation kinetics of dimer and trimer were biphasic and the rate of dissociation was accelarated by high concentrations of monomer.

Project Description:

Objectives: (1) To develop an understanding of the relationship between cell surface events and the biochemical pathway which a cell follows. In the basophil system this means sorting out the parameter domains corresponding to specific desensitization, non specific desensitization, histamine secretion, and inactivity. (2) To develop a detailed understanding of Fc receptor redistributions upon the binding antigen-antibody complexes. (3) To determine and study the relation between cluster size and distribution and cellular activity. (4) To develop an understanding of the relationship between the thermodynamics and kinetics of ligand cell-bound receptor interactions, and the requirements for lymphocyte activation.

Methods Employed: Mathematical modeling; mathematical analysis of experimental data.

Major Findings: Basophils sensitized to penicillin degranulate and release histamine when incubated with multivalent penicillin derivatives but not with monovalent hapten. The dose-response curve is biphasic with maximal release at a concentration of dimeric hapten of about 1 nM. The characteristics of the response generated using mixtures of monovalent and divalent derivatives, as well as a variety of other evidence, suggest that the rise and fall in the dose-response curve reflects the rise and fall in the concentration of receptors cross-linked by the multivalent hapten.

Extracellular Ca^{2+} is required for histamine release and cells may be densensitized to different degrees by incubation with various concentrations of ligand in the absence of Ca^{2+}. Washing and rechallenging cells with an optimal dose of ligand and Ca^{2+} indicates that the dose dependence of desensitization is also biphasic and is most pronounced at a ligand concentration which ordinarily stimulates maximal release. The implication is that cross-linking, which, for the thermodynamic parameters characteristics of this system, will lead primarily to dimers and trimers, initiates signals for both degranulation and desensitization.

Kinetic studies reveal release curves which are sigmoidal, having delays in the onset of release that vary with ligand concentration. The magnitude of the delay is a biphasic function of concentration with a maximum at approximately the same concentration as the peak in the dose-response curve. The results are interpreted in terms of a model in which cross-linked receptors are converted to an active, unstable intermediate which facilitates an increase in cytoplasmic Ca^{2+}, but which decays spontaneously into an inactive product. Dependence of histamine release on the concentration of the intermediate is nonlinear, suggesting either a positive feedback loop stabilizing the intermediate or the interaction of several aggregates. A fit of a simple mathematical formulation of the model indicates that it qualitatively and quantitatively explains the dose-response, desensitization, and release patterns.

Scatchard plots for IgG oligomers are convex to the origin. The result provides the first direct experimental evidence that receptor clustering leads to such plots. The rate of dissociation of labeled ligand is faster in the presence of cold ligand than in its absence. The result again provides the first direct experimental evidence that receptor clustering can cause such accelaration. Both effects are widely observed in cell systems, and they have

previously been attributed to negative binding cooperativity caused by conformational changes in the receptor. The results therefore may require a reassesment of the interpretation of a wide variety of data. The method in principle should be applicable to any cell that responds via a receptor clustering mechanism. For such systems it is important to know whether failure of the cell to respond at high ligand concentrations is simply the result of inability to cross-link (because all receptor sites are saturated) or the result of some desensitization signal induced by large aggregates. We have currently identified AgE and human basophils as a system in which a desensitization signal prevents release at high concentrations.

Significance to Biomedical Research and the Program of the Institute: Aside from the obvious bearing on allergic reactions, the basophil system, because it is a one ligand-one cell system which responds in minutes, permits the type of quantitative analysis which is not possible for antibody production and secretion. It is therefore a model system for studying cellular activation.

This project is related to the projects on lymphocyte activation, complement activation and insulin binding. For the first two, binding of antibodies to cells via the Fc receptor must precede biological activity. A detailed understanding of the nature of the complexes formed is a necessary component of developing an understanding of the biological processes. The problem of determining and quantitating the parameters that effect the initial states of signal transduction in basophils is an important aspect of the attempt to understand the nature of the immediate allergic reaction. More generally, however, the basophil system may serve as a model from which to build a deeper understanding of the relation between the binding of ligand to cell surface receptors and subsequent biochemical events and factors that regulate them.

Proposed Course: Experimental tests of many of the predictions are now in progress. The model will be extended to include non specific desensitization.

The theory makes predictions related to the effect of variations in the mean number of cell surface receptors which will be tested experimentally. The relation between binding and activity under various conditions will be determined and analyzed.

Publications:

Chabay, R. DeLisi, C., Hook, W. A., and Siraganian, R.: Receptor cross-linking and histamine release in basophils. J. Biol. Chem. 295: 4628-4635, 1980.

Perelson, A., DeLisi, C., and Siraganian, R.: A method for determining whether the descending limb of biophasic dose response curve reflects insufficient cross-linking. Molec. Immunol. In press.

Dower, S.K., DeLisi, C. Titus, T.A., and Segal, O.M.: The mechanisms of binding of multivalent immune complexes to Fc receptors I: Equilibrium studies. Biochemistry. In press.

Dower, S.K., Titus, J.A., DeLisi, C., and Segal, D.M.: The mechanism of binding of multivalent immune complexes to Fc receptors II: Kinetics of binding. Biochemisty. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08341-03 LMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Physical Chemical Studies of Lipid-Protein Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John N. Weinstein, M.D., Ph.D.	Investigator	LMB	NCI
OTHERS:	Robert P. Blumenthal, Ph.D.	Chief, Membrane Structure & Function Section	LMB	NCI
	Richard Klausner, M.D.	Research Associate	LMB	NCI
	Evelyn Ralston, Ph.D.	Visiting Associate	LMB	NCI

COOPERATING UNITS (if any) T. Innerarity and R. Pitas, Univ. of California at San Francisco; A. Wlodawer, National Bureau of Standards; J.P. Segrest, Univ. of Alabama at Birmingham; J. Goerke, Univ. of California, San Francisco; Robert Guy, Armed Forces Research Institute

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have investigated the interaction of lipoproteins with liposomes to form recombinant particles. A number of lipoprotein fractions (VLDL, IDL, LDL, and HDL) all disrupt liposome structure by an essentially irreversible and quasi-stoichiometric process. In the case of HDL, the major apoprotein, A-I, recombines with dimyristoyl phosphatidyl choline vesicles at 40:1 lipid:protein to form discs approximately 100 Å in diameter and 32 Å in thickness, with protein on the rim. These structural results were obtained by a combination of neutron scattering, electron microscopy, and column chromatography.

With dipalmitoyl phosphatidylcholine, A-I also forms what we term "vesicular recombinant" particles in a process which may relate to physiological mechanisms by which proteins are assembled into membranes and lipoproteins. To study this process we have developed a technique called "phase transition release" (PTR) which is also being applied to study incorporation of tubulin into membranes.

Lipoproteins were labelled with the fluorescent lipid 3,3-dioctadecylindocarbocyanine for studies of interaction with cell surface lipoprotein receptors.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To investigate the interaction between liposomes and lipoproteins and between lipoproteins and cells. More specifically,

(1) To define the mechanism by which liposomes are broken down by serum components, principally the lipoproteins;

(2) To determine the relative efficacies of different purified apolipoproteins in breaking up liposomes;

(3) To develop fluorescently labelled lipoproteins and liposome-apoprotein recombinants for use in studying lipoprotein-cell interactions;

(4) To use the methods developed for objectives (1) - (3) to assess the physical chemistry of protein-lipid interaction in lipoproteins;

(5) To extend to other bilayer-protein interactions the concepts thus developed;

(6) In particular, to investigate the assembly of tubulin and actin into membranes.

Non-standard Methods Employed: (a) Preparation of liposomes, by probe and bath-sonication; (b) Dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory, i.e., "phase transition release" (PTR); (c) Determination of the mobility of lipoproteins and liposomes bound to the cells, using fluorescence-photobleaching recovery; (d) Neutron scattering studies of lipoprotein size and shape; (e) Aqueous collisional fluorescence quenching studies of lipid-protein interaction; (f) Derivatization of proteins with trinitrobenzene sulfonate; (g) labelling of lipoproteins with 3,3'-dioctadecylindocarbocyanine (diI) dye.

Major Findings: (1) Liposomes are broken down and their contents released by an interaction with serum, most rapidly at the lipid phase transition. (2) The serum effect is mostly due to low density (LDL) and high density (HDL) lipoproteins, but very low density and intermediate density lipoproteins also play a part, as also does some other, unidentified component of the serum. (3) Heparinized plasma, EDTA plasma, and serum all have the same effect. (4) At least in the case of apo-HDL the mechanism appears to be a quasi-stoichiometric, all-or-nothing breakdown of the vesicle into a small disc of lipid rimmed with protein. The process is largely complete within a second or two at the phase transition of the liposome lipid and is essentially irreversible on that time scale. (5) By neutron diffraction and electron microscopic studies the discs appear to be about 100 Å in diameter and 32 Å in thickness, with a protein rim. They probably consist of a single bilayer. (6) Cholesterol at 40 mole percent in the liposomes severely restricts the interaction with serum components, as does the formation of liposomes from lipids which are below their phase transitions at the temperature of study. (7) At molar ratios of about 2,000:1 dipalmitoyl phosphatidylcholine:Apo A-I, a stable vesicular recombinant particle (VR) is formed below the lipid phase transition temperature (Tc). As the temperature is raised through Tc (in PTR), a new type of recombinant (VR-Tc) is formed. By physical measurements of several types, the A-I's conformation and disposition in the lipid change at Tc. The protein probably becomes transmembrane. These findings may relate to physiology processes for formation of

HDL and to the assembly of intrinsic proteins into membranes. (8) Purified tubulin interacts with vesicles at Tc to form vesucular recombinants. The interaction is accompanied by structural changes in lipid and protein. (9) HDL, LDL, and apoE-HDL can all be labelled efficiently and irreversibly with the fluorescent lipid analogue, diI. The lipoproteins are unchanged in physical properties and in specific binding to cell surface receptors. The labelled lipoproteins were acetoacetylated for studies of phagocytic and lipoprotein-specific uptake in arterial walls. (10) Studies of the interaction with bilayers of a hepatic membrane receptor for asialoglycoprotein are described in another report (ZO1 CB 08343-01 LTB).

Significance to Biomedical Research and the Program of the Institute: (1) A major barrier to the effective use of liposomes as carriers in cancer chemotherapy and diagnosis has been a lack of understanding of their interactions with serum. Our studies define the interaction and indicate what type of liposomes must be used to avoid it. (2) Our studies of HDL-liposome recombinants contribute to (1) and may also be useful in delineating mechanisms of atherosclerosis. Our fluorescently labelled lipoproteins are currently being used by collaborators to study atherogenesis. (3) Breakdown of liposomes in serum is essential to the combination of liposomes with hyperthermia to achieve selective release of drugs in the area of a tumor.

Proposed Course: We are continuing work on most of the objectives listed. Liposome-lipoprotein interactions are being studied by phase transition release in combination with other physical and biochemical techniques; lipoproteins and liposome-lipoprotein recombinants are being fluorescently labeled for studies of their physical chemistry and interactions with cells; the requirements for a serum-stable liposomal carrier are being defined, with emphasis now on the differences among different size liposomes; neutron scattering studies will be extended to higher angle regions; electron micrographs of HDL-liposome recombinants will be subjected to image analysis to enhance detail; assembly of tubulin into bilayers and the possible physiological correlates will be investigated in further detail; the fluorescent labelling of lipoproteins has been achieved and will be continued in our laboratory.

Publications:

Weinstein, J.N., Magin, R.L., Cysyk, R.L., and Zaharko, D.S.: Treatment of solid L1210 murine tumors with local hyperthermia and temperature-sensitive liposomes containing methotrexate. Cancer Research. 40: 1388-1395, 1980.

Klausner, R.L., Bridges, K., Tsunoo, H., Blumenthal, R., Weinstein, J.N., and Ashwell, G.: Conformational changes in the hepatic asialoglycoprotein receptor upon interaction with membranes and ligand. Proc. Nat. Acad. Sci. 77: 5087-5091, 1980.

Magin, R.L., and Weinstein, J.N.: Selective delivery of drugs in "temperature-sensitive" liposomes. In Tom, B.H. and Six, H.R., (Eds.): Liposomes and Immunobiology. Amsterdam, Elsevier, 1980, 315-325, 1980.

Blumenthal, R., Klausner, R.D., Weinstein, J.N., Bridges, K., Tsunoo, H., and Ashwell, G.: Voltage-dependent translocation of hepatic binding protein across lipid model membranes. Annals N.Y. Acad. Sci. 358: 368-369, 1980.

Guo, L.S.S., Hamilton, R., Goerke, J., Weinstein, J.N. and Havel, R.J.: Interaction of unilamellar liposomes with serum lipoproteins and apolipoproteins. J. Lipid Research. 21: 993-1003, 1980.

Blumenthal, R., Klausner, R.D., and Weinstein, J.N.: Voltage-dependent translocation of the asialoglycoprotein receptor across lipid membranes. Nature. 288: 333-338, 1980.

Weinstein, J.N., Klausner, R.D., Innerarity, T.L., Ralston, E., and Blumenthal, R.: "Phase transition release" (PTR), a new approach to the interaction of proteins with lipid vesicles: application to lipoproteins. Biochim. Biophys. Acta. In press.

Pitas, R.E., Innerarity, T.L., Weinstein, J.N., and Mahley, R.W.: Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. Atherosclerosis. In press.

Klausner, R.D., Kumar, N., Weinstein, J.N., Blumenthal, R., and Flavin, M.: Interaction of tubulin with phospholipid vesicles II: physical changes of the protein. J. Biol. Chem. In press.

Klausner, R.D., Kumar, N., Weinstein, J.N., Blumenthal, R., and Flavin, M.: Interaction of tubulin with phospholipid vesicles I: association with vesicles at the phase transition. J. Biol. Chem. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08342-02 LMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Theory of Receptor-ligand Biophysics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Charles DeLisi, Ph.D. Senior Investigator LMB NCI

COOPERATING UNITS (if any)
Dr. Alan Perelson, Theor, Div. Los Alamos National Lab., Los Alamos, NM; Prof. Frederik Wiegel, Dept. of Physics, Twente Univ. of Tech., Enschede, Netherlands; Prof. Federico Marchetti, University of Rome, Rome Italy

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rate constants for ligands interacting with cell bound or dispersed receptors have a diffusive part and an intrinsic part: the former depending on geometry, receptor distributions, and diffusion coefficients; the latter on electronic redistributions. We have been focusing on the former and have obtained expressions for diffusion limited association and dissociation rate constants when (1) ligands bind directly and specifically to receptors that are distributed over a spherical surface; (2) ligands bind indirectly by a path that includes non specific association with the cell and diffusion in the surface, toward or away from a specific receptor. We have also developed a formalism that permits calculation of the complete equilibrium and rate constants for cell bound receptors, given the equilibrium or rate constants for dispersed receptors.

Mathematical methods are also being developed to describe aggregation on a two dimensional fluid surface.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: To obtain expressions that can be used to analyze kinetic data for reactions between ligands and cell bound receptors. To obtain analytic expressions for the time evolution of the distribution function describing the growth of aggregates subsequent to the binding of ligand to laterally mobile cell surface receptors.

Major Findings: The kinetic problem for the entire distribution function, with and without loop closure (cyclic complexes) was solved exactly for the first time for a bivalent-bivalent system. Because the formulation of the problem leads to an infinite system of coupled non-linear equations, numerical solutions are not possible. The analytical results thus allow applications that were previously not possible.

The theory is being used to answer a variety of questions of biological interest. One of its applications, the analysis of ligands dissociating from cell surface receptors, predicts that accelerated dissociation by cold ligand and multi exponential decay of label, is expected even in the absence of negative cooperativity or cross-linking. The conditions under which such observations are expected have been derived, and a consideration of known rate constants indicates that they are likely to be met in a large number of cases.

Clustering of receptors is responsible for cell activation and/or desensitization in a variety of systems. Moreover, since very few clusters - perhaps three or four - are needed to induce activity in some systems the question of how cells prevent spontaneous activation arises. Equilibrium calculations based on the entropic loss in constraining receptors to be adjacent, indicate that hundreds of pairs are expected on cells having 10^5-10^6 receptors.

The results of this project shed light on this problem. The main idea is that receptors must remain within some minimum distance of one another for some required amount of time to tranduce a signal. The equations indicate that receptors will diffuse out of this required distance very rapidly, on the order of 10^{-5} seconds. Thus for transduction times of the order of a millisecond, the ratio of separation time to transduction time (the central parameter) is in the range of 10-100. One can then show that the probability of spontaneous transduction can easily be as low or lower than 10^{-30}. With even a weak affinity ligand, the probability changes to close to one. Thus a control mechanism based on a residence time requirement can easily lead to virtually infinite amplification of transduction.

Significance to Biomedical Research and the Program of the Institute: The methods are required to develop an understanding of the physical chemistry of the regulation of cell responsiveness at the cell surface level. Aspects of these ideas may be relevant to a wide range of biological processes including enzymic enhancement of catalysis. The results will also allow correlations to be drawn about cellular activity and the size of the aggregates which form on the cell surface, i.e., previously it was possible only to connect the mean number of crosslinked receptors with activity; now the importance of the way they are distributed in various sized aggregates can be assessed. This project is a necessary aspect of all other research projects.

Proposed Course: Additional fundamental work on estimating the accuracy with which a cell can sense its environment; applications to a variety of dose response data including lysozomal enzyme release, histamine secretion, chemoreception and complement activation. Applications to basophils and mast cells responding to multifunctional antigens. Assessement of the role of valence and geometry in the regulation of the response.

Publications:

DeLisi, C.: The biophysics of ligand-receptor interactions. Quart. Rev. Biophys. 13: 201-230, 1980.

Wiegel, F., and DeLisi, C.: Effect of non-specific forces and finite receptor number on ligand-cell bound receptor rate constants. Proc. Nat. Acad. Sci. In press.

DeLisi, C.: The effect of cell size and receptor density on receptor-ligand rate constants. Molec. Immunol. 18: 507-511, 1981.

DeLisi, C.: Theory of the clustering of cell surface receptors by ligands of arbitrary valence: Dependence of dose response patterns on coarse cluster characteristics. Math. Biosci. 52: 159-184, 1980.

Pincus, M., DeLisi, C., and Rendell, M.: Ligand binding to multiple equivalent sites with steric hindarance. Biophys. Biochem. Acta. In press.

DeLisi, C., and Perelson, A.: Toward a dynamical theory of membrane organization and function. In DeLisi, C., Perelson, A., and Wiegel, F. (Eds.): Physical Chemistry of Cell Surface Phenomenoa. Marcel Dekker. In press.

DeLisi, C.: The magnitude of signal amplification by ligand induced receptor clustering. Nature. 289: 322-323, 1981.

Thakur, A., and DeLisi, C.: Fluctuation analysis in small chemically reacting systems: conductance noise in membrane channels. Bull. Math. Biol. 42: 147-160, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08343-02 LMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Movement of Molecules Across Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.P. Blumenthal, Ph.D.	Chief, Membrane Structure and Function Section	LMB NCI
OTHERS:			
	John N. Weinstein, M.D., Ph.D.	Investigator	LMB NCI
	Richard D. Klausner, M.D.	Research Associate	LMB NCI
	A. Joseph Van Renswoude, M.D., Ph.D.	Visiting Fellow	LMB NCI
	Christoph M. Kempf, Ph.D.	Visiting Fellow	LMB NCI

COOPERATING UNITS (if any)

P. Henkart, Ph.D., T. Lewis Ph.D.	IB NCI
G. Ashwell, Ph.D., C. Steer, M.D.	LBM NIAMDD
W.H. Habig, Ph.D.,	BB DBP

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Membrane Structure and Function Section

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1.5	1.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Electrical properties of bimolecular lipid membranes (BLM) are measured to study: (1) mechanisms of ion transport; (2) properties of transport systems isolated from natural cell membranes; (3) mechanisms of cytotoxicity; (4) the effect of the membrane potential on the disposition of membrane proteins. We have continued our studies with the hepatic asialoglycoprotein receptor (the hepatic binding protein, or HBP), which induces voltage-dependent increases in ion conductance across a BLM, and is probably translocated across the bilayer under the influence of the electric field in the presence of specific ligand or $Ca2^{+}$. These observations suggested a mechanism by which biological membranes might regulate the disposition of their proteins. We have carried out a theoretical analysis on the way electrostatic forces operating on charge clusters, found in transmembrane proteins, may determine the final orientation of the protein. To test the predictions of that analysis we are studying the topology with respect to the bilayer of mellitin, a peptide with a hydophobic domain and a positively charged domain of amino acids. Under the influence of a trans-negative membrane potential mellitin induces an increase in BLM conductance, which is sensitive to pronase. We are also studying the interaction of clathrin with BLMs.

Project Description:

Objectives: To study the physical mechanisms of ion transport in reconstituted membranes. To develop the lipid bilayer membrane as an assay for transport systems materials isolated from natural cell membranes. To study mechanisms of cytotoxicity. To study the effect of the membrane potential on the disposition of membrane proteins.

Methods Employed: The bilayer membranes are formed from natural membrane extracts, oxidized cholesterol, or pure lipids in an aperture between two electrolyte solutions. The electrical properties of the membranes are measured before and after application of an activating factor. Spectroscopic changes upon interaction with lipid vesicles are studied by fluorometry and circular dichroism.

Major Findings: (1) Histograms of conductance step increases of HBP in a BLM showed a wide distribution around a peak step-size, suggesting that the increase is caused by a lipid perturbation. (2) The voltage-dependent conductance induced by HBP can be titrated reversibly by lowering the pH on the cis side of the membrane. (3) The lower pH probably did not cause the bilayer to become more resistant to lipid perturbation, because other lipid perturbers such as clathrin did not fail to induce conductance at lower pH. (4) Measurements of transport of radiolabelled ligand for HBP across a HBP-doped BLM failed to show specific HBP-mediated transport; this suggests that a binding site for ligand is translocated without attached ligand. (5) Under the influence of a trans-negative membrane potential mellitin induced a conductance increase across a BLM, which could be destroyed by adding pronase to the trans side. This finding suggests that the conductance increase is caused by movement of a positive group (lysine or arginine) across the bilayer. (6) An analysis of the role of charge clusters in membrane proteins on their orientation in the membrane showed that a) transmembrane proteins, for which information is available, have a cluster of positively charged amino acids at the cytoplasmic end of the transmembrane hydrophobic segment, and a cluster of negatively charged amino acids at the extra-cytoplasmic end, and b) electrostatic forces, which include the transmembrane potential as well as the surface potential and the dipole potential, may determine the final orientation of the inserted protein.

Significance for Biomedical Research and the Program of the Institute: Understanding membrane transport and mechanisims of cytotoxicity are considered to have important implications for cell transformation and for many aspects of the physiology of normal and tumor cells.

Proposed Course: The BLM will continue to be used as an assay for conductance-inducing materials from cells. The notion of voltage-dependent assembly of proteins in membranes will be further explored with other proteins and using biochemical techniques. The nature of the conductance change induced by the studies on cell-mediated cytotoxicity will be pursued, using cytotoxic T-cells.

Publications:

Blumenthal, R., and Klausner, R.D.: The Interaction of Membrane Proteins with Black Lipid Membranes. In Poste, G. and Nicholson, G.L. (Eds.): Cell Surface Reviews. Amsterdam, Elsevier/North-Holland vol. 9, in press.

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Klausner, R.D., Bridges, K., Tsunoo, M., Blumenthal, R., Weinstein, J.N., and Ashwell G.: Physical changes in the hepatic asialoglycoprotein receptor upon interaction with membranes and ligand. Proc. Natl. Acad. Sci., USA. 77: 5087-5091, 1980.

Blumenthal, R., Klausner, R.D., Weinstein, J.N., Bridges, K., Tsunoo, H., and Ashwell, G.: Voltage-dependent translocation of hepatic binding protein across lipid model membranes. Ann. N.Y. Acad. Sci. In press.

Blumenthal, R., Klausner, R.D., and Weinstein, J.N.: Voltage-dependent transloction of the asialoglycoprotein receptor across lipid membranes. Nature (Lond.) 288: 333-338, 1980.

Blumenthal, R., Klausner, R.D., and Weinstein, J.N.: Voltage-dependent changes of a membrane protein in lipid model membranes: studies with the hepatic asialoglycoprotein receptor. Biophys. Disc. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08345-02 LMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Conformational and Pumping Studies of Bacteriorhodopsin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard D. Klausner, M.D.	Research Associate	LMB NCI
OTHERS:	S. Roy Caplan, Ph.D.	Visiting Scientist	LMB NCI
	Alan Kleinfeld, Ph.D.	Biophysical Lab, Harvard Med.	School, Boston
	John N. Weinstein, M.D., Ph.D.	Investigator	LMB NCI
	Robert Blumenthal, Ph.D.	Chief, Membrane Structure and Function Section	LMB NCI
	Mones Berman, Ph.D.	Chief	LMB NCI

COOPERATING UNITS (if any)
None

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.3	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Bacteriorhodopsin (BR) is a 27,000 dalton protein that can be isolated from the purple membrane of Halobacterium halobium. It functions as a light driven proton pump. Crystalline purple membrane fragments are isolated, solubilized by detergent and reconstituted into pure egg yolk lecithin vesicles. Tryptophan fluorescence reveals a highly blue shifted emission spectrum whose quantum yield is unchanged with light-dark adaptation. Tryptophan lifetime heterogeneity analysis reveals two populations of fluorophores, one of which is altered by light adaptation. Fluorescence energy transfer from BR tryptophan to anthrostearic acid probes inserted into the vesicles allows us to map the spatial distribution of the tryptophans and sensitively monitor conformational changes in the protein. This is a new technique that will allow in situ conformational analysis of membrane proteins. Studies of the detailed pumping kinetics of BR reconstituted into lipid vesicles has allowed us to model the processes involved in pumping of protons.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To develop the application of the technique of energy transfer to the study of membrane protein conformation. To study the conformation of BR and correlate conformational changes with its proton-pumping action.

Methods Employed:

1. Reconstitution: Several techniques for replacing detergent with defined lipids to form BR-containing vesicles are used including dialysis and exchange chromatography.
2. Spectroscopy: Circular dichroism is used to monitor retinal-retinal excitons as a measure of the aggregation state of the protein. Tryptophan fluorescence with analysis of the tryptophan lifetimes to define the environmental heterogeneity of these amino acids in the protein.
3. Fluorescence energy transfer: stearic acid is conjugated to anthracene and used as the acceptor for the non-radiative energy transfer from tryptophan flourescent donors. Each probe used has the anthracene moiety attached to a specific carbon of the fatty acid acyl chain. The amount of energy transfer (measured by the quenching of tryptophan fluorescence) from BR to each of these probes is determined.
4. Energy transfer rates can be calculated from the anthracene probes to the retinal and the retinal can therefore be localized.
5. The validity of the general technique of energy transfer from multiple tryptophans to these probes are tested in two ways:

   A. Experimentally

      1) Carefully measuring the overlap integrals of the tryptophans and each probe.

      2) Measuring the decay anisotropy of each probe in these vesicles to obtain a measure of the relative contributions of orientation and distance to the energy transfer rates.

      3) Measuring the effects of the different probes on the lifetime distribution of the tryptophans.

   B. Theoretically

      Computer modeling of the range of geometric configurations compatible with the energy transfer calculations. This will allow us to establish limits on the sensitivity of the method to conformational changes.

6. Monomeric and multimeric BR-containing vesicles are irradiated with light of controlled intensity and the kinetics of proton movement followed. Theoretic models for describing these kinetics are derived.

Significant Findings: 1. Conditions for the reconstitution of BR as a monomer or in a multimeric form have been developed. 2. A three compartment model has been proposed for proton translocation. This postulates a proton uptake by the protein as a reflection of light-induced conformational change and a true pumping process. 3. There are two populations of tryptophan in BR: one with a lifetime of .7 nsec. and another minor component with a lifetime of 7 nsec. Light adaptation does not change the overall quantum yield of BR tryptophan fluorescence but does shorten the minor lifetime component to 5 nsec. 4. Energy transfer suggests that there are two masses of tryptophans, one located near the surface of the membrane and the other located about 15A outside of the membrane on the opposite side to the first massing. Light adaptation of the BR produces a specific alteration of the energy transfer profile suggesting a shift in the massing of the tryptophan.

Significance for Biomedical Research and the Program of the Institute: The ability to analyze conformation changes in membrane proteins is essential to understanding the normal and aberrant behavior of cell membranes.

Proposed Course:

1) To specifically test the pumping model by:
   a) Looking for spectroscopic evidence of the proposed conformational change.
   b) Comparing the observed kinetics of pumping under different perturbing conditions to the predictions of our model.
2) To arrive at a compatible molecular model for the structure of BR with our energy transfer data.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08348-02 LMB

PERIOD COVERED: October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Receptor-ligand Kinetics and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mones Berman, Ph.D.	Chief	LMB NCI
Other:	David Covell, Ph.D.	Staff Fellow	LMB NCI
	Jerry Gardner, M.D.	Chief	DD NIAMDD
	Ruth Chabay, Ph.D.	Staff Fellow	LMB NCI

COOPERATING UNITS (if any)

Digestive Diseases Br., NIAMDD

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several receptor systems are being investigated by modeling the hormone kinetics, binding and function. Hormones investigated include insulin, glucagon, secretogogues and others.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To develop a general, unified hormone-receptor model from the insights gained by studying the individual systems.

Major Findings: The kinetics of cellular calcium have been investigated in response to secretogogues (Chabay) and other stimulators (Covell). Modeling in this area has progressed slowly.

Significance to Biomedical Research and the Program of the Institute: The receptor systems are most important in the control of cellular processes and are thus relevant to much of the biological research conducted in this and other laboratories at NIH and elsewhere.

Proposed Course: Development of the model using various receptor systems in-vitro and in-vivo will continue.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08349-01 LMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Macromolecular Conformational Characterization with Electro-optic Methods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert L. Jernigan, Ph.D.	Theoretical Physical Chemist	LMB NCI
OTHERS:	Sanzo Miyazawa, Ph.D.	Visiting Fellow	LMB NCI

COOPERATING UNITS (if any)

None

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0.5	0.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Kerr Effect is an unusual physical property because it depends on both the molecule's dipole moment and its polarizability tensor. The particular quantity that must be averaged over all conformations is the square of the dipole moment times the polarizability tensor. For rigid rods, this yields a dependence on the third power of the molecular weight; whereas for a random coil its average will depend only on the first power. More common characterization methods would display second or first power dependences. These dependences were recently verified in reported experiments on DNA restriction fragments. Calculations for helix-coil transitions of polypeptides indicate that Kerr Effect measurements would be significantly more sensitive to the early stages of helix formation than are other methods. In addition, the Kerr Effect is extremely sensitive to an individual peptide's conformation. The method should provide useful information about the formation of conformations of anisotropic shape.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To determine the potential utility of electric birefringence measurements (Kerr Effect) in distinguishing among macromolecular conformations. In particular, a major question is how effective the method would be in monitoring helixcoil transitions.

Methods Employed: Statistical mechanical methods of averaging the appropriate physical quantitites, involving dipole moments and polarizability tensors. Statistical descriptions of macromolecular conformations were employed. For polypeptides, statistical mechanical descriptions of the helix to random coil transition were utilized.

Major Findings: The Kerr Effect is potentially most useful in characterizing the conformations of macromolecules which have substantial dipole moments. For non-polar macromolecules, induced dipole-induced dipole interactions may be substantial. Formalism was developed to treat such interactions. Results of applying this method to hydrocarbons yield good agreement with experiments. For a single peptide residue, the values of the Kerr constant for the usual accessible conformations range from 0 to -100 cm^5 statvolt^{-2} mol^{-1} x 10^{-12} for beta conformations to about 1000 for an alpha helix. This large range indicates a sensitivity to conformation substantially greater than for most other properties that have been studied. The molecular weight dependence of the Kerr constant for rigid helices is M^3 whereas for most other commonly measured properties it is only M^2 or M^1. The molecular weight dependence of all such properties for random coils is the first power of M. Recently reported experiments on DNA restriction fragments have confirmed these dependences. For double stranded DNA, short fragments evidence a cubic dependence, converging to a first power dependence for higher molecular weights, as flexibility appears. This change in molecular weight dependence makes the method more sensitive to the onset of rigidity than other methods. It provides an especially useful tool for investigating the formation of anisotropic macromolecular shapes, because it can monitor earlier stages of their formation than can other more common physical properties. Our calculations suggest that Kerr Effect measurements may be particularly useful in investigations of helix-coil transitions.

Significance to Biomedical Research and the Program of the Institute: Basic experimental tools to monitor changes in macromolecular shape are needed for better understanding of molecular function in molecular biology.

Proposed Course: Calculations of Kerr constants could be applied to DNA solutions to study helix flexibility and to protein folding to indicate the potential for detecting anisotropic intermediates.

Publications:

Jernigan, R. L., and Miyazawa, S.: Kerr effects of flexible macromolecules. In Krause, S. (Ed.): Molecular Electro-optics. New York, Plenum Press, 1981, pp. 163-179.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08351-01 LMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Studies on the Folding and Secretion of Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard D. Klausner, M.D.	Research Associate	LMB NCI
OTHERS:		
A. Joseph Van Renswoude, M.D. Ph.D.	Visiting Fellow	LMB NCI
Robert P. Blumenthal, Ph.D.	Chief, Membrane Structure and Function Section	LMB NCI
John N. Weinstein, M.D., Ph.D.	Investigator	LMB NCI
Christoph M. Kempf, Ph.D.	Visiting Fellow	LMB NCI
Dinah Singer, Ph.D.	Immunology Branch	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Membrane Structure and Function Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.6	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

One of the major problems of secretion is understanding the mechanisms whereby a secreted protein is translocated across the lipid bilayer. The energetic problems of this process are formidable. We are taking two approaches to the problem: 1) to define whether we can trap and isolate folding intermediates of secretory proteins in vitro to define whether they pass through hydrophobic states that may explain their interaction with and translocation across the membrane. Specifically, we are studying ovalbumin, the major secreted protein of chicken eggs. This protein was chosen for study because, unlike most secreted proteins, it is not synthesized as a precursor protein with an additional hydrophobic leader sequence. 2) We are also studying how to couple the in vitro synthesis of secreted proteins such as immunoglobulin and ovalbumin from mRNA to membranes using rough endoplasmic reticulum as an acceptor membrane. By using this approach, we hope to define the structures of the membrane that allow the recognition and translocation of secreted proteins.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To define the biophysical and biochemical bases of protein secretion and to examine in particular, the relationship between folding and this process.

Methods Employed: 1) Protein folding: a number of spectroscopic and labelling techniques are used to study this process including fluorescence, circular dichroism, and absorption spectroscopy. In addition, a series of hydrophobic affinity probes are used to identify the presence of hydrophobic conformers as well as to localize the portion of the protein exposed in these altered forms. 2) Standard techniques of in vitro protein translation from isolated mRNA are employed. Purified subcellular membranes are coupled to the translation system and chromatographic or density gradient centrifugation techniques are used to look for association and sequesteration of newly synthesized proteins within the membrane vesicles.

Significant Findings: 1) Protein folding: a) Ovalbumin, when denatured in vitro, does not return to its native form upon renaturation but assumes a new form called OAR. b) OAR has been characterized as distinct from native ovalbumin by spectroscopic, chemical and immunologic techniques. c) OAR has definite hydrophobic characteristics, including the binding of lipids and hydrophobic molecules, interaction with lipid bilayers and association with hydrophobic affinity resins. d) The hydrophobic site can be covalently labelled and shown to be limited primarily to one tryptic fragment which we are currently identifying. This fragment may well correspond to a proposed internal signal sequence that may be essential to the secretion of ovalbumin.

2) Synthesis-secretion coupling: Preliminary evidence suggests that we are indeed able to sequester newly synthesized proteins into rough endoplasmic reticulum vesicles.

Significance to Biomedical Research and the Program of the Institute: These studies will hopefully elucidate the mechanism that allow and control secretion of proteins and how these processes may be manipulated and altered by membrane alterations and defects.

Proposed Course:

1) With the results so far obtained we hope to be able to identify the hydrophobic sequence (s) of ovalbumin and determine what role it plays in ovalbumin secretion. Using antibody specific to the two forms of ovalbumin, we can test which form is initially synthesis by the ribosome in the presence and absence of membranes. We will do this by isolating chicken oviduct mRNA for ovalbumin and study its in vitro synthesis in a cell free translation system.

2) By coupling the in vitro synthesis of secreted proteins to membrane vesicles, we hope to dissect those membranes to understand when structures are required for translocation. These studies will include attempts to identify specific sites for translocation and the role of membrane lipids and lipid structure in this process.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08352-01 LMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Synthesis and Insertion of a Membrane Protein

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard D. Klausner, M.D.	Research Associate	LMB NCI
OTHERS:		
A. Joseph Van Renswoude, M.D., Ph.D.	Visiting Fellow	LMB NCI
Robert Blumenthal, Ph.D.	Chief, Membrane Structure and Function Section	LMB NCI
John N. Weinstein M.D., Ph.D.	Investigator	LMB NCI
Dinah Singer, Ph.D.	Immunology Branch	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Stucture and Function Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.1	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Very little is known about the genetic organization, transcription, translation and insertion of integral plasma membrane proteins. Bacteriorhodopsin is a very well characterized integral membrane protein produced by Halobacterium halobium. H. halobium is grown under conditions that either do or do not lead to the induction of bacteriorhodopsin. Techniques for successfully isolating and characterizing ribosomal and mRNA are compared and appropriate in vitro translation systems are tested for the ability to translate H. halobium message, in general, and bacteriohodopsin messenger in particular. The latter is identified by immunoprecipitation with specific antibodies which have been developed. From this starting point, the gene, messenger and initial translation product for bacteriorhopsin will be chracterized. The ability to insert the in vitro synthesized peptide will allow us to determine the characteristics and, hopefully, the mechanism of protein insertion and membrane assembly.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To develop a system in which the gene, messenger and gene product for an integral membrane protein-bacteriorhodopsin can be examined. To define the requirements for the insertion and assembly into the membrane of this protein.

Methods Employed: a) Bacterial cultures of H. halobium. b) Isolation of mRNA by modifications of standard regimes. c) Development of a cell free translation system from H. halobium to compare the ability of such a system to translate H. halobium mRNA with an E. coli system and a eukaryotic sytem. d) Isolation and characterization of membrane vesicles from H. halobium to serve as acceptor membranes. e) Standard techniques of molecular cloning.

Significant Findings: 1) H. halobium message can be isolated intact from the bacterium. 2) This message can be, at least partially, translated in a eukaryotic system. 3) Translation products include several discrete protein bands on polyacrylamide gel electrophoresis.

Significance to Biomedical Research and the Progaram of the Institute: If successful, these studies will provide a unique system for studying the genetic controls over membrane protein synthesis and allow us to dissect the process of membrane insertion. Clearly, if the membrane indeed proves to be a significant locus of the expression of the tranformed state, understanding how the genome, mRNA, nascent peptides and the membrane interact will be invaluable.

Proposed Course:

1) We will continue to isolate and characterize the mRNA fron H. halobium.
2) We will attempt to identify a system in which the messenger coding for bacteriorhodopsin can be translated.
3) From this point we will concentrate two projects:
   a) How the translated membrane protein is inserted into acceptor membranes.
   b) Attempts to isolate and clone the gene for this membrane protein.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08353-01 LMB

PERIOD COVERED: January 1, 1981 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Perpetuation and Eradication of Viruses in Large Populations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Ira B. Schwartz, Ph.D. Staff Fellow LMB NCI

COOPERATING UNITS (if any)

Professor James A. Yorke, IPST, University of Maryland, College Park, Maryland

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We examine the role of seasonal variation in transmissibility of epidemic models. In particular we consider deterministic models for viruses in large populations and show that seasonal fluctuation in the incidence is necessary for undamped perpetation of the virus. Extensive applications are made to the measles virus via computer simulations.

PHS-6040
(Rev. 2-81)

Position Description

Objectives: We attempt to find the underlying mechanisms of seasonality in viruses that sustain undamped recurrent outbreaks of viral infections. In addition, we are developing efficient software for solving numerically a general class of epidemic models.

Methods Employed: Computer modelling; mathematical analysis; numerical analysis.

Major Findings: It is shown that for human virus infections such as measles, poliomyelitis and hepatitus B, seasonality plays a major role in the perpetuation of diseases. Extensive computer simulations demonstrate in the case of measles that there exist many periodic oscillations which range from small to very large fluctuations in the number of infectives. The existence of large fluctuating periodic oscillations leads to short terms of irregular oscillations in the number of cases of disease as is reported in Baltimore in the pre-vaccine era from 1928-1950.

Signifance for Biomedical Research and the Program of the Institute: Eradication is the converse of perpetuation and represents the ultimate methods for control of an infectious disease. To determine the potential for eradication, it is necessary first to understand the requirements for perpetuation. Thus, the subject is hightly relavent to practical goals in public health and preventive medicine.

Publications:

Schwartz, I.B.: Estimating regions existence of unstable periodic orbits using computer-based techniques. SIAM Journal on Numerical Analysis. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08354-01 LMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Enzyme-Substrate Complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Matthew R. Pincus, M.D., Ph.D. Expert, Theoretical Chemist LMB NCI

OTHER: Sandra Smith-Gill, Ph.D. IPA LCB NCI

COOPERATING UNITS (if any)

Professor Harold Scheraga, Cornell University, Ithaca, NY

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.25	PROFESSIONAL: 0.25	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Molecular recognition underlies most biological processes, but it is a phenomenon which is not well understood. Recently, potential energy functions have been developed that can be used to accurately calculate the conformational energies of molecules. These conformational energy calculations can be applied to the interactions of biological macromolecules, with other molecules. By examining biomolecular complexes of lowest free energy and by determining which interactions are the critical ones in stabilizing the complex, the basis for molecular recognition can be understood. We are currently using such conformational energy calculations in studies of enzyme-substrate complexes including lysozyme and other hydrolytic enzymes.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To calculate the three-dimensional structures of enzyme-substrate complexes using conformational energy calculations; to test predicted structures experimentally, and to determine the critical interactions involved in the best structures. Applications are to lysozyme which shows strong specificity for its substrates, and which is a simple enzyme for experimental study. The ultimate object is to predict the structures of molecules that bind with specific affinities to drug receptors.

Methods Employed: Conformational energy calculations are used to compute all allowed conformations for the isolated substrate and the enzyme-substrate complex. Multi-dimensional phase space searches are employed to determine allowed binding regions for simpler substrates. The structures of all complexes determined in this step are then subjected to energy minimization, allowing for all internal and external degrees of freedom for the substrate and internal degrees of freedom for the enzyme. We have recently predicted the structure of an enzyme-hexasacchraide complex. We are currently using monoclonal antibodies to a specific calculated subsite to test whether the substrate acutally binds to this specific site as calculated.

Major Findings: We have calculated unique, lowest energy structures for a variety of -1,4-linked N-acetylglucosamine polymers bound to the active site of the enzyme. These structures agree quite well with experimentally determined structures on the basis of X-ray crystallography. We have computed new binding sites not previously thought to be involved in the binding of saccharides to the active site of the enzyme; in particular, a different D site from the one proposed on the basis of crystallographic model-building and a new F site on the side of the cleft opposite Arg 114 (as proposed on the basis of model building), involving such residues as Arg 45, Asn 46, and Thr 47. The former prediction has been fully verified by X-ray crystallography. We are currently testing the latter calculation in the following novel way. Recently, a monoclonal antibody has been raised to lysozyme that binds quite specifically to our calculated F site, i.e., to residues 45, 46, and 47. Preliminary results indicate that hexasaccharides do indeed bind with their terminal sugar residue in this site.

We have further calculated the structures of copolymers of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), which are the natural cell wall substrates for this enzyme. We have calculated that specific interactions between the lactic acid side chain in site F make the alternating copolymer the best substrate for the enzyme, in agreement with solution studies.

Significance to Biomedical Research and the Program of the Institute: Since our calculations have been successful in predicting structures of enzyme-substrate complexes, we can now attempt to determine which molecules would offer optimal interactions and serve as either substrates or inhibitors. Such studies can suggest molecules that might act as effective drugs.

Publications:

Pincus, M.R., and Scheraga, H.A: Prediction of the three-dimensional structures of complexes of lysozyme with cell wall substrates. Biochemistry. In press.

Pincus, M.R., and Scheraga, H.A: Theoretical calculations on enzyme-substrate complexes. The basis of molecular recognition and catalysis. Acc. Chemical Res. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08355-01 LMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Binding of Multivalent Antibodies to Antigen Lattices

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Matthew R. Pincus, M.D., Ph.D., Expert, Theoretical Chemist LMB NCI

COOPERATING UNITS (if any)

Dr. Marc Rendell, M.D., Assistant Professor, Department of Medicine, The Johns Hopkins Medical Center

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The most sensitive assay for antibodies is the solid phase radioimmunoassay in which picomolar quantities of antibody can be accurately detected by binding to antigens attached to a solid phase. It would be highly desirable to obtain from such assays, the intrinsic affinities of the antibody for the antigen, the size of the antibody, and its valency. Previous theoretical approaches to this problem have assumed that the lattices are "dilute" containing at most two antigens. However, the antigens may be closely packed so that excluded volume effects, where adjacent lattice sites are blocked, become important to the binding. The fact that each antibody is multivalent, i.e., can bind to more than one antigen, further complicates the overall process. Thus, any theory of antibody binding to antigen lattices should include the effects of many antigen sites, the effects of excluded volume and the multivalency of the antibody.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To obtain a general expression for the binding of di- and multivalent antibodies to antigen lattices. This expression relates the fraction of antibody bound to the amount of free antibody present, and accounts for excluded volume and multivalency. The validity of the derived expression is being tested experimentally over a wide range of antibody concentration.

Methods Employed: Statistical mechanical methods are employed to obtain the grand partition function for the system. For a one dimension lattice, matrix generation of the partition function yields analytical solutions. In two concentrations, Monte Carlo techniques are applied to obtain the partition function. Once the partition function is known, the requisite saturation function is obtained directly.

Major Findings: A general expression for the binding saturation function has been derived from a solution of the generalized one dimensional Ising model which effects of neighboring antigens sites upon the antigen state. The solution of the Ising model directly yields the partition function from which all thermodynamic properties of the system can be calculated. By fitting the parameters in the theory to experimental data, one can obtain the individual intrinsic affinity constants for the binding of each "arm" of the antibody to the lattice, the number of sites excluded on the binding of each antibody arm, size of the antibody and the number of antigen sites on the lattice.

This model has been generalized to include the effects of variable span length between individual antibody "arms" and variable numbers of excluded sites occur upon or binding individual antibody "arms". We are also currently performing experiments to test the range of validity of the model.

Significance to Biomedical Research and the Program of the Institute: The expressions derived thus far can now be used to analyze antibody binding data in which solid phase methods are used, including affinity chromatography. These should permit a more thorough understanding of immunological assays.

Proposed Course: The one dimensional treatment will be extended to two dimensions. This problem is being approached in two ways. First, a solution to divalent antibody binding to the two-dimensional lattice is being sought by using transfer matrices. Second, a Monte Carlo generation of all possible bindings at various antibody concentrations is being developed in order to obtain numerical evaluation of the partition function. We will compare the results with solid phase radioimmunoassay data on insulin antibodies to test the theory.

Publications:

Pincus, M.R., DeLisi, C., and Rendell, M.: Ligand binding to multiple equivalent sites with steric hindrance. Biochim. et Biophys. Acta. In press.

Pincus, M.R. and Rendell, M.: General quantitative treatment for the binding of divalent antibodies to solid phase immunobilized antigens. Proc. Natl. Acad. Sci., USA. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08356-01 LMB

PERIOD COVERED: October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Use of Kalman Filtering in the Design of Optimal Therapeutic Drug Strategies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: David Covell, Ph.D. Staff Fellow LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .01	PROFESSIONAL: .01	OTHER: 0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A computer program will be developed to determine optimal drug dosage schedules. An extended Kalman filter will be employed to recursively update noise statistics from measurements of the system being investigated. The resultant statistics will be used to determine dosage schedules and/or detect changes in the characteristics of the patient.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To improve the information yielded from data analyzed in pharmacokinetic protocols.

Methods Employed: The information yielded from data is compromised by statistical inaccuracies. Such "noise" includes analytical (measurement) and state (system) variation. The identification of these noise statistics leads to improved understanding of the system's performance. The theory of Kalman filtering permits such identification.

The results of the filtering analysis serve a dual purpose. The statistical information can be used to judge the "significance" of an observed state and/or measurement deviation. This information is useful for determining whether the deviation represents a a serious (possibly pathological) change or whether the deviation is acceptable for the population within which the patient is classified. The second consequence of the identification analysis is to permit direct application of optimal control theories towards improved drug scheduling. This includes suggesting optimal times for drug administration and sampling as well as the mode of drug administration (i.v. vs p.o./bolus vs infusion) to achieve the "best" treatment.

Major Findings: The proposed analysis provides a means for evaluating the practicalities of using a kinetic model for suggesting drug schedules. The value of this analysis lies in the ability to assimilate information about a system's performance and to apply this knowledge towards testing current methods of drug treatment and suggesting possible means of improvement.

Significance to Biomedical Research and the Program of the Institute: Better drug scheduling will result in greater patient safety and improved drug efficacy.

SUMMARY REPORT
IMMUNOLOGY BRANCH
October 1980 - September 1981

The Immunology Branch carries out laboratory investigations in basic immunobiology with particular emphasis in the following areas: 1) Regulation and control of immune responses; 2) Structure and function of cell surface molecules; 3) Transplantation biology; and 4) Tumor immunology including clinical studies in immunotherapy. In addition, the Immunology Branch maintains a fluorescence activated cell sorter facility which is involved integrally in many of the studies carried out in the Branch in each of the above areas, and is also used in a large number of collaborative investigations with other laboratories at NIH. In this summary report I shall touch briefly on the highlights of accomplishments in each of these areas during the past year, giving references where appropriate to the individual annual reports in which more detailed information on these accomplishments can be found.

A. REGULATION AND CONTROL OF IMMUNE RESPONSES

Work in Dr. Hodes' laboratory has been directed at studies of the generation and regulation of T cell dependent responses to both conventional and alloantigens, and at the mechanisms of interaction among T cells, B cells and accessory cell populations. During the past year studies have characterized the T cell subpopulations involved in the generation and regulation of cytotoxic T lymphocytes (CTL) directed toward H-2 different stimulators (CB-05055). It was demonstrated that the generation of CTL requires precursors which are Lyt 1^+ 23^+ T cells, and that the differentiated effectors are also Lyt 1^+ 23^+. A distinct T cell subpopulation which was phenotyped as Lyt 1^+ 23^- differentiated in vitro into a regulatory population capable of suppressing the generation of CTL precursors. These findings provide a basis for further analyzing the interaction of T cell subpopulations in regulation of immune response.

In addition, a number of collaborative studies concerning genetic control of the immune response to staphylococcal nuclease have been carried out as a collaboration between the laboratories of Drs. Hodes and Sachs. The in vitro antibody response to TNP-nuclease has been shown to be under the control of immune response (Ir) genes in the I-B region, and the function of such genes is expressed at least in part at the level of antigen-presenting or accessory cells (CB-05086). Since no serologically detectable I-B encoded Ia antigens have been described, these findings raise questions concerning the nature of the I-B gene product expressed by accessory cells. The function of Ia antiens in Ir gene expression has been further evaluated in both T cell dependent antibody responses and antigen-specific T cell proliferation. Monoclonal anti-Ia reagents, including those specific for gene complementation products of I-A and I-E subregions, inhibit in antigen-specific fashion the T cell recognition of antigen, consistent with a direct or steric relationship between Ia antigens and Ir gene products.

Studies in Dr. Sachs' laboratory, also involving genetic control of the immune response to nuclease, have been directed toward examination of the in vivo effects of treatment with anti-idiotypic antibodies (CB-05036). Injection of

pig anti-idiotypic antibodies into virgin mice has been found to lead to an increase in idiotype levels in the serum of these mice. The idiotype detected was predominantly found on immunoglobulin molecules without antigen binding activity for nuclease. Similar treatment of nude mice did not lead to idiotype expression in serum, suggesting that the effect might involve T cells. In collaboration again with Dr. Hodes' laboratory, studies were performed to examine the expression and functional involvement of idiotype on T cells in a system of T cell dependent antibody responses to TNP-nuclease (CB-05092). Nuclease-primed helper T cells of a given mouse strain express determinants identical to or cross-reactive with those expressed on nuclease-specific antibodies produced by the same inbred strain. Antibody responses to TNP-nuclease were inhibited by anti-idiotype in vitro, and this effect was mediated by an effect upon helper T cell function, supporting the concept that idiotypic determinants expressed by T cells are important to the function of those cells. Anti-idiotype administered in vivo was shown to result in the generation of idiotype-bearing nuclease-specific primed helper T cells, demonstrating the ability of anti-idiotype to trigger these cells in the absence of antigen. Moreover, a given anti-idiotype was able to induce idiotype-positive helper T cells in strains of mice which do not express that idiotype in responses to the antigen nuclease, emphasizing the importance of regulatory effects in determining idiotype expression.

Drs. Hodes and Singer have also begun to study monclonal T cell populations specific for the antigens (T,G)-A--L and KLH (CB-05086). Cloned (T,G)-A--L specific Lyt 1^+ T cells were competent and highly efficient in providing T cell help for in vitro antibody responses. Such T cells were both H-2-restricted and antigen-specific, demonstrating the ability of a single T helper cell population to recognize both antigen and self H-2.

Studies in the laboratory of Dr. Al Singer have been directed towards investigations of genetic control of immune cell interactions among macrophages, T cells and B cells. In the last year it has been shown by anti-Ia inhibition experiments that the I region encoded restricting elements which helper T cells recognize are the Ia antigens macrophage express (CB-05064). It has also been shown in experiments with fully allogeneic low responder --> responder radiation bone marrow chimeras that the Ir gene phenotype of helper T cells perfectly parallels their ability to recognize as "self" the Ia determinants of the high responder haplotype. Thus, these two series of experiments are the strongest evidence to date that the Ia antigens are the products of Ir genes.

In another series of experiments, it was shown this year that developmentally distinct B cell subpopulations are activated by helper T cells in genetically distinct ways. The activation by T cells of Lyb5- B cells is MHC restricted while the activation of $Lyb5^+$ B cells is not. Furthermore, the genetically unrestricted mechanism by which Lyb5+B cells are activated by T cells was shown to derive from their unique capacity to be triggered by soluble T cell factors. These experiments not only make possible the further functional study of B cell subpopulations, but also suggest a resolution to a long standing controversy in cellular immunology over whether or not T-B interactions are genetically restricted.

In additional studies in Dr. Singer's laboratory it has been shown that all thymic independent type II antigen responses required Ia^+ macrophages (CB-05090). These results have now created the foundation for determining whether or not TI-2 responses are genetically restricted.

Ongoing studies in Dr. Gene Shearer's laboratory have been directed toward a) understanding the role of the major histocompatibility complex in regulation and restriction of T cell mediated and effected immune responses against chemical haptens and infectious viruses (CB-05038) and b) the genetic and mechanistic aspects of natural resistance to graft-vs-host (GvH) reactions (CB-05088). In the past year a new series of hapten-self-specific CTL responses have been developed in the mouse (CB-05038 and CB-05098). In contrast to our previous work involving haptenic reagents which covalently couple to amino groups on cell surface proteins, these new hapten are conjugated to free sulfhydryl moieties. Most interesting has been the observation that all eight of the sulfhydrylreactive haptens thus far tested exhibit greater H-2 restriction than do the amino-reactive series of haptens and show the opposite pattern of Ir gene controlled high and low responsiveness compared to the amino reactive series of haptens. These results suggest that CTL effector cell specificity is strongly influenced by the haptenic portion of the foreign antigen-self immunogenic complex, whereas the pattern of Ir gene control is greatly and possibly exclusively determined by the self determinant recognized.

In studies in collaboration between Drs. Steven Shaw and Bill Biddison in Dr. Shearer's laboratory, genetic control of human CTL responses against influenza-virus infected autologous cells have been carried out (CB-05078). These studies have demonstrated that a particular donor's T cells preferentially recognize virus in association with products of only one of the two possible parental haplotypes. Such preferential recognition of self HLA appears to be virus-specific, since type A and type B influenza viruses are often recognized in association with different haplotypes by the same donor. HLA-A2 and HLA-A3 variants have been found which are indistinguishable from wild type -A2 and -A3 by serological means but are different by CTL studies against virus-self. Cellular analysis studies suggest that human CTL responses against influenza-self require helper T cells and Ia positive macrophages. The types of studies outlined here are of value in elucidating the role of HLA antigens in regulating human T cell immunity against infectious virus and of defining the cellular components involved.

B. STRUCTURE AND FUNCTION OF CELL SURFACE MOLECULES

Studies in Dr. Pierre Henkart's laboratory have continued to probe molecular features of membrane damage induced by immune mechanisms (CB-05018). Previous studies of marker permeability of red cell ghost membranes demonstrated the sieving properties expected of membrane pores induced by cytotoxic lymphocytes, complement, and low concentrations of detergent. In the past year this approach has been extended to liposomes. Marker sieving in liposomes treated with low concentrations of several detergents has been detected, making it clear that previous findings were not somehow dependent on red cell membrane structure. In addition, progress has been made in setting up a system to study the action of cytotoxic T lymphocytes on artificial planar lipid bilayer membranes.

Work in the laboratory of Dr. Howard Dickler has continued to probe mechanisms involved in the triggering and regulation of immunocompetent cells, and the role that cell surface molecules play in this triggering. In the past year, characterization of receptor interactions on the surface of B lymphocytes has been greatly extended and solidified (CB-05035).

It is now clear that several distinct membrane receptor interactions take place upon binding of ligand to receptors. These include: interaction between the receptor for the Fc portion of IgG antibody (FcGR) and the antigen receptor surface IgM; interaction between FcGR and the antigen receptor surface IgD; and interaction between FcGR and immune response (Ir) gene associated (Ia) antigens (which may be the product of Ir genes). Each of these interactions is specific and unique in its characteristics. The involvement of FcGR in each of these interactions provides substantive evidence for a central role for this receptor in B lymphocyte function. Based on these findings a hypothetical scheme for the regulation of B cell function has been constructed. Currently, functional assays have been developed and are being utilized to evaluate the physiologic importance of receptor interactions and to test the aforementioned hypothesis.

Work in Dr. Dickler's laboratory has also been directed toward interactions which may take place on cell surfaces via idiotype-anti-idiotype recognition (CB-05058). A system has been developed which allows in vitro production of antibody responses to an antigen (T,G)-A--L (the response to which is Ir gene regulated). The characteristics of this response in vitro are identical to those of in vivo responses, and idiotype can be detected on such antibody. In addition, anti-idiotype reagents have been developed as probes. Since these latter reagents detect both strain-specific and cross-reactive idiotypes, work is in progress to absorb the cross-reactive idiotypes leaving strain-specific probes.

Studies in Dr. David Segal's laboratory have been directed toward understanding the molecular and cellular basis of the interactions of immunoglobulins with immune effector systems, and the relationship of antigenic recognition to these interactions (CB-05050). Techniques have been developed for measuring the binding of model immune complexes to Fc receptors on cells. Using these methods, kinetic studies have been performed yielding quantitative information on immunologically relevent binding at the cell surface. Binding and release processes have been found to have both fast and slow components. The biphasic nature of these kinetics was not predicted by the simple multivalent mechanism, nor was it a result of affinity heterogeneity of ligand binding, since none was observed. It has therefore been suggested that the slow binding and release reactions result from some of the receptors being in environments on the cell surface which are less accessible to medium than others. The mechanistic implications of these findings are being further studied. Other studies in Dr. Segal's laboratory have employed flow microfluorometry to quantitate receptors and other molecules on the cell surface. Fc receptor densities on different subpopulations of human cells have been studied, and the internalization of such receptors following binding of immunoglobulin polymers has been examined.

Morphologic studies of cellular interactions in immune systems have recently been initiated by Dr. Maryanna Henkart (CB-05102). Studies in Dr. Henkart's laboratory have been directed toward electron microscopic examinations of cell populations involved in cell mediated killing. As a first example to be studied, Dr. Henkart has chosen human natural killer (NK) cells and has studied them in serial thin sections. Characteristic granules containing bundles of tubules in crystalline arrays have been found. Within an hour of binding to target cells tubule-containing granules appear to fuse and the contents of the fused granules are secreted into the space between killer and target cells.

The secreted products cling to and may fuse with the target cell membrane. These findings may therefore be relevant to the mechanisms of NK cell killing. Similar studies have been initiated for other populations of immune-related killer cells. Dr. M. Henkart has also continued her characterization of intracellular calcium-containing organelles in a variety of cell populations (CB-05096).

C. TRANSPLANTATION BIOLOGY

Studies in Dr. David Sachs' laboratory have been directed toward understanding of the structure and function of products of the major histocompatibility complex, and manipulations of the immune response to these products (CB-05021). In the past year, a large number of hybridoma cell lines producing antibodies to H-2 and Ia antigens have been produced and characterized. These antibodies have been used to further subdivide products of the MHC. In addition, anti-idiotypic antibodies against these hybridomas have been produced and the effects of such anti-idiotypic reagents on in vitro and in vivo parameters of histocompatibility have been examined. Treatment of animals with the anti-idiotypic antibodies has been found to induce appearance of idiotype in the serum. A percentage of the induced idiotype has been shown to bear the same anti-H-2 or anti-Ia specificity as the original monoclonal antibody. These findings therefore represent the induction of anti-H-2 and anti-Ia antibody responses in the absence of antigen exposure. These anti-idiotypic antibodies may thus provide an approach to modification of the immune response to MHC antigens.

In addition, studies of transplantation biology in the miniature swine model have been continued (CB-05023). Milligram quantities of histocompatibility antigens have been prepared from individual pig spleens, and N-terminal amino acid sequences have now been obtained for the SLA antigens of two of the three partially inbred lines of miniature swine. Comparisons of these sequences with each other and with the sequences of MHC antigens from other species reveal high levels of homology, as well as possible allotypic differences. Two new recombinants within the MHC have been detected within the miniature swine herd. Both recombinants involve separation of the MLC stimulatory locus (SLA-D) from the serologic loci (SLA-ABC). Transplantation studies aimed at determining the relative importance of individual MHC loci are now in progress using these new recombinant lines.

Studies in Dr. Hodes' laboratory have examined the nature of the alloreactive T cell repertoire (CB-05087). This repertoire has been studied in proliferative mixed lymphocyte responses (MLR) to non-H-2 linked Mls products and in the generation of CTL specific for products of mutant K^b genes. T cells were shown to recognize Mls determinants in the context of the self H-2 determinants with which they are presented, thus resembling H-2 restricted T cell recognition of conventional (non-allo) antigens. Using radiation bone marrow chimeras, it was found that the T cell repertoire employed in CTL respones to mutant K^b products is influenced by the environment in which those T cells mature. These findings are of fundamental importance in suggesting that the T cell repertoire for alloantigens, like that for conventional antigens, may be both MHC restricted and environmentally modified.

The use of radiation bone marrow chimeric animals promises to provide a valuable approach to discerning the mechanism of generation of cell mediated immune responses to transplantation antigens. Studies in Dr. Al Singer's laboratory this past year have demonstrated that the self-recognition repertoire of thymocytes is absolutely determined by radiation-resistant thymic elements (CB-05094). Since T cells normally first become functionally competent in the thymus, these experiments demonstrated that the T cell self-recognition repertoire was determined either prior to or concomitant with the acquisition of functional competence in the thymus.

In other studies in Dr. Singer's laboratory, the possibility that T cell precursors expressed receptors prior to their migration into the thymus was examined by determining whether T cell precursors could be specifically tolerized by the pre-thymic environment (CB-05093). In the past year it was shown that T cell precursors are specifically tolerized by their pre-thymic environment. Thus, these experiments suggest that T cell precursors express their receptors for allogeneic determinants prior to their entry into the thymus. These experiments have important implications for current understanding on how the T cell receptor repertoire is generated.

Dr. Shearer's laboratory has continued its interest in graft-vs-host disease (CB-05049 and CB-05088). Studies this year have shown that mice can be protected from the immunosuppressive effects of chronic GvH disease when they are injected with a variety of antisera specific for different cell surface antigens (both H-2 and non-H-2). Exposure to these sera appears to activate regulatory cells which prevent immunosuppression.

Studies in the laboratory of Dr. Stephen Shaw are directed toward the nature of human T cell recognition and activation, with a particular emphasis on the genetics of the human major histocompatibility complex (HLA). The major emphasis this past year has been on defining a new HLA gene, designated "SB" or "secondary B cell" gene (CB-05101). Population studies indicate that the five defined antigens are part of a single segregant series of antigens in Hardy/Weinberg equilibrium. These antigens are only weakly associated in the population with the other B cell antigens: HLA-DR. Proof that these antigens are products of a new locus was obtained in family studies (two of which show segregation of SB independent of other HLA gene products) and in studies of mutant lymphoblastoid cell lines (which showed that DR could be lost without loss of SB).

Studies have also been initiated by Dr. Shaw on the relevance of these new SB markers to the diseases multiple sclerosis and dermatitis herpetiformis (CB-05100). In the 38 unrelated MS patients studied, there was no significant abnormality in the frequency of SB antigens. However, among the 41 DH patients studied, there was a significant elevation of the frequency of the SB1 antigen and a decrease in the frequency of the SB2 antigen. These data cannot be explained by the known strong association between DH and DR3; rather they indicate that there is an interaction between the HLA-SB and the HLA-DR phenotype in determining the risk for dermatitis herpetiformis.

D. MOLECULAR BIOLOGY

Dr. Dinah Singer's laboratory has been involved in studies of the organization of genes encoding the major histocompatibility complex and mechanisms controlling the expression of these genes (CB-05083). During the past year, using recombinant DNA technology, genomic fragments containing MHC genes of both mouse and pig have been isolated. Analysis of these clones demonstrates that individual MHC genes are separated by at least 10 kilobases of structurally unrelated DNA. Analysis of total DNA from both species reveals that there are multiple segments of homologous DNA, perhaps 3-4 times more than accounted for by known MHC antigens. Both intra-species and inter-species differences in genome organization of these genes has been observed by partial DNA sequence analysis.

Studies on the regulation of gene expression have been directed at isolating and characterizing RNA species encoding MHC antigens. In both the mouse and miniature swine, mRNA species encoding MHC antigens have been identified. In addition, in the miniature swine, a novel RNA species containing sequences homologous to MHC sequences has been found. A coupled system of in vitro translation and processing is being developed to study the role of post--translational processing in the regulation of MHC antigen expression.

E. TUMOR IMMUNOLOGY

Studies in Dr. John Wunderlich's laboratory have been directed toward identification of factors which influence host cytotoxic cell responses against syngeneic tumors (CB-05003). A fully syngeneic in vitro system for generating primary mouse cytotoxic cell responses detected by 4 hour chromium release assays with MCA-induced tumor target cells has been established. The antitumor cytotoxic cell activity has been shown to be mediated by $Thy1^-$, $Lyt2^-$ NK^+ cells. Thus the effector cells are a type of natural killer (NK) cell. However, in vitro generation of these anti-tumor NK cells and their activity differ significantly from that which occurs naturally in vivo (classic NK) in that 1) Different patterns of activity occur when classic NK cells and in vitro induced NK cells are tested against a panel of target cells, and 2) Spleen cells from both beige mice and young mice (1-4 weeks) have relatively little classic NK activity but generate normal or higher than normal levels of NK activity in vitro. Therefore, in vitro stimulation appears to result in a subpopulation or different population of NK cells compared to those occurring naturally in vivo.

NK effector cells induced in vitro with poly-I primarily recognized a common determinant on widely diverse target cells or else expressed multiple receptors of different specificities at the single cell level. Of note, freshly dispersed cells from primary MCA-induced tumors were also susceptible target cells. There was little indication of effector cell polyclonality based on target cell recognition. Tumor cells cocultured with unprimed spleen cells modulated but did not induce anti-tumor cytotoxic cell responses. Inducers included poly-I, Con-A induced spleen cell supernatants, and fetal calf serum. Studies of the in vitro anti-tumor cytotoxic cell responses indicate that they may be controlled by multiple non-H-2 genes.

Clinical trials of immunotherapy in the treatment of human malignant melanoma have been continued (CB-05033). The studies involve a controlled, randomized comparison of immunotherapy to chemotherapy in Stage I and Stage II malignant melanoma. Patients have been randomly assigned to receive treatment either with methyl CCNU, BCG alone or BCG plus allogeneic tissue-culture-grown vaccine. A fourth group consists of control patients who receive no further active treatment. A total of 181 patients have entered the trial, and patient accrual has been terminated. Studies of the peripheral blood lymphocytes of patients in this clinical trial indicated that greater than 90% of those receiving the cell vaccine generated cellular and/or humoral immune responses against one or more of the vaccine cell lines. In addition, all patients in the study had a normal capacity for generating cytotoxic cellular responses in vitro except for those patients receiving chemotherapy who showed some depression in this functional capacity. However, no significant differences between treatment groups have been detected to date.

F. FLUORESCENCE T ACTIVATED CELL SORTER

The Immunology Branch has continued to maintain an active Fluorescence Activated Cell Sorter Facility operated by Ms. Susan Sharrow (CB-05062). The Fluorescence Activated Cell Sorter (FACS) has been used both to analyze and to separate lymphoid populations by rapid flow microfluorometry. Cell suspensions are first treated with appropriate fluorescent reagents and then subjected to the FACS analysis. Studies performed in this facility have been an integral part of many of the investigations described in the above sections. In addition, numerous collaborative studies with other investigators at NIH and elsewhere have been performed.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05003-16 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Cytotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Wunderlich	Senior Investigator	I NCI
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COOPERATING UNITS (if any)

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3.0	1.5	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Broadly reactive antitumor cytotoxic cell activity can be induced in vitro in normal mouse spleen cells by polyinosinic acid, supernatants of Con A-stimulated cells or fetal calf serum. Like primary in vitro responses to allogeneic transplantation antigens, activity against syngeneic tumor cells normally peaks only after 4-5 days of culture. Syngeneic irradiated sarcoma cells alone only modulate and will not induce such activity. The effector cells appear to recognize a common determinant on widely diverse target cells or else express multiple receptors of different specificities at the single cell level. The effector cells do not react with normal lymphoid cells. Freshly dispersed tumor cells from primary MCA-induced sarcomas are also susceptible to these syngeneic effector cells, and the tumor cells are not injured by natural killer cells from syngeneic nude mouse spleens. Spleen cells from individual mice from crosses involving high and low responder strains have been tested. Analysis of parents, F1's and backcross mice, indicates that responsiveness is under the control of multiple dominant or codominant genes.

Project Description

Objectives: This project has been directed at understanding and manipulating mechanisms of cell-mediated immune cytotoxicity in both humans and mice. Long-range goals are: (1) to identify and characterize factors which influence induction, maturation and expression of cytotoxic cellular immune responses; (2) to define changes in cytotoxic cellular immunity which occur in tumor bearing hosts; and (3) to find means of manipulating the cytotoxic cellular immune response for therapeutic purposes.

Methods Employed: Cell-mediated cytotoxicity is measured in vitro by release of ^{51}Cr from labelled target cells incubated with lymphoid cells for 2-6 hours. Immune cells are collected from spleen, marrow, lymph node, thymus and peripheral blood of sensitized hosts. Cells have been fractionated by 1 g velocity sedimentation, density gradient separation in Percoll, removal of cells which ingest iron particles, removal of cells which adhere to anti-Ig coated petri dishes and lysis of cells by monclonal antibodies and complement. Hybridoma cell lines producing monclonal antibodies have been obtained from the Salk Cell Distribution Center and recloned. In addition effector cells have been produced by in vitro sensitization of lymphoid cells using previously established Mishell and Dutton tissue culture conditions. Broadly reactive effector cells have been generated primarily by culturing normal mouse spleen cells with polyinosinic acid in medium supplemented with syngeneic plasma. Target cell lines have been adapted to growth in medium supplemented with mouse plasma, this medium also can be used to support cytotoxicity assays. Target cells are provided by freshly explanted cells (including primary MCA induced tumor cells dispersed with highly purified collagenase and DNAse), tissue culture lines and established tumor cell lines passed in vivo or in vitro; most target cells function well if previously frozen in liquid nitrogen. Target cells also have been chemically modified in order to change the immunogenicity of cell surface antigens.

Sarcomas have been induced with methylcholanthrene (MCA) in mice which are routinely screened for potentially immunsuppressive pathogens before admission to the colony at the Frederick Cancer Research Center. Tumor cell lines established from these sarcomas are also screened for potentially immunosuppressive pathogens and contaminating virus-related antigens: mycoplasma (culture and serology) and a variety of viruses (serology) including MVM, Sendai, LDH and LCM. The tumor cell lines used in the study were induced by a relatively low dose of MCA (100ug) and do not stimulate generation of anti-tumor cytotoxic cells under standard in vitro conditions for generating cytotoxic cells against allogeneic transplantation antigens.

Major Findings: Previous work in this laboratory demonstrated that normal mouse spleen cells cultured for 5 days in medium supplemented with syngeneic plasma and polyinosinic acid (Poly I) generate theta-bearing cytotoxic cells, whose broad pattern of target cell reactivity includes syngeneic tumor cells. A similar pattern of cytotoxicity develops if syngeneic sarcoma cells are included in the "sensitization" mixture. This effort has been extended by the following findings.

Different factors have been identified which induce in vitro generation of broadly reactive cytotoxicity. Normal mouse spleen cells cultured alone in medium supplemented with syngeneic plasma do not develop cytotoxic activity. Two additives other than Poly I have been identified which will induce such activity: fetal calf serum (FCS), and supernatants from Con A-stimulated spleen cells. Con A in the supernatants is subsequently blocked by alpha-methylmannoside; supernatants of control cultures pulsed with Con A are inactive. FCS-induced activity is detected against target cells grown and tested in medium supplemented with mouse serum to minimize the role of FCS antigens. Spleen cells from all mouse strains tested generate cytotoxic activity in response to FCS, but only a few strains respond to Poly I with high activity. Thus, a high responder strain, C57BL/6(B6), has been used for most of our work with poly I induced cytotoxicity. Of note, the Poly I induced response from B6 cells starts to rise only after two days of culture and peaks at 4-5 days, which is not consistent with Poly I acting directly as an interferon inducer. We have not been able to induce cytotoxicity with syngeneic irradiated chemically-induced sarcoma cells alone. However, inclusion of tumor cells in spleen cell cultures containing poly I or Con A-induced supernatant augments the generation of cytotoxic cell activity. Thus, tumor cells will modulate but will not induce in vitro generation of broadly reactive cytotoxic cells.

Target cell specificity of Poly I-induced effector cells has been further tested. Cytotoxic activity has been inhibited with unlabeled target cells in order to determine the basis for the broad reactivity pattern induced by Poly I. Marked cross-reactivity has been observed using diverse sources of unlabeled target cells, including human melanoma cells, syngeneic and allogeneic sarcoma cells, and hamster fibroblast cells. Media supplemented with plasma syngeneic to the responding cells have been used for generation of activity, for growing target cell lines and for the assay itself in order to eliminate the possibility of a common contaminating antigen. Cytotoxic activity of Poly I-induced effector cells has not been observed against syngeneic or allogeneic mitogen-induced lymphoblasts. Thus, the broad reactivity of Poly I-induced effector cells is selective and cannot be explained by a simple polyclonal expansion theory. Instead, many effector cells appear to recognize a determinant shared between tumor cells and cultured cell lines, or else the effector cells express multiple receptors with individual specificities at the single cell level.

Tumor cells dispersed by highly purified DNAse and collagenase from freshly explanted primary MCA induced sarcomas have also been used as target cells for Poly I-induced cytotoxic cells. Tumor cells isolated on the day of the assay are susceptible to such activity generated by syngeneic effector cells; but the tumor cells are not susceptible to fresh spleen cells from syngeneic nude mice which have high levels of classic NK activity. Syngeneic lymphoblasts similarly treated with enzymes are not susceptible to poly I induced effector cells. Both types of target cells are susceptible to appropriate allogeneic effector cells. These observations are important because tumor cells adapted to growth in tissue culture can change their expression of cell surface determinants and their susceptibility to immune lysis. Thus, the present studies

indicate that primary tumor cells are susceptible. Further work is needed to address the possibility that immune activity is directed against infiltrating host cells and thus serves in a regulatory role.

Genetic control of the generation of broadly reactive anti-tumor cytotoxic cells has been analyzed by testing individual donors from high (C56BL/6) and low (BALB/c) responder parents, their F_1 (B6xBALB) progeny and the F_1xBALB backcross progeny in multiple experiments. All crosses intentionally involved high responder females because of positive maternal influence on the response. Scoring mice as high or low responders by formal cluster analysis gave no unexpected results among 22 parental mice and 2 unexpected results among 11 F_1 mice. The frequency of high responders among 38 backcross mice is too low to be compatible with single gene control, in keeping with studies of the genetic control of NK activity in situ. Although the number of mice in the present work is low, the methodology appears adequate and additional testing will be done. Multiple testing of Bailey's CXB inbred recombinant mice which are descended from randomly mated pairs of an F_2 generation from C57BL/6 x BALB/c, has revealed low and high responder strains. This information will be useful in pursuing linkages of the regulating genes.

Significance to Biomedical Research and the Program of the Institute: Broadly reactive anti-tumor cytotoxic cells induced in vitro in this study are similar in many ways to natural killer (NK) cells and to cytotoxic cells found in tumor bearing patients. The present in vitro system offers a model for identifying factors which regulate the generation of such activity. The influence of tumor cells, presensitization of donor mice and genetic control are of particular interest. The combination of properties of the cytotoxic cell response under study here which make it unique are that 1) the response develops from unprimed spleen cells in the absence of histoincompatible cells or foreign serum and thus is a model for a "primary" anti-tumor response, 2) the time course of the response is not consistent with interferon-induced augmentation of preexisting NK cells, 3) the reactivity pattern of cytotoxic cells is selective and does not include fresh normal cells and 4) the broad pattern of reactivity does not result simply from polyclonal expansion of target-specific effector cells. Moreover, broadly reactive anti-tumor effector cells generated in vitro are cytotoxic for freshly excised syngeneic primary sarcoma cells and are the first type of syngeneic effector cell we have found which is cytotoxic for such target cells. Finally, they do not react with normal cells in contrast to certain other types of broadly reactive cytotoxic cells, e.g. those induced by allogeneic effect factors.

Proposed Course of Project: The following directions are planned for this project:

1. Antisera against classic T and NK cell surface determinants will be used to compare NK cells and broadly reactive anti-tumor cytotoxic cells generated in vitro. We have produced anti-asialo GM1 which is highly active against NK cells, and generation of allogeneic anti-NK 1.2 is in progress. Monoclonal antibodies against Lyt determinants are now generally available.

2. Analysis of the target cell specificity of broadly reactive anti-tumor cytotoxic cells has been limited to tests for direct cytotoxicity and inhibition of cytotoxicity. Generation of effector cell clones under limiting dilution conditions during the first five days of culture has not been reliable using syngeneic conditions, probably because of insufficient ancillary cell function. We will circumvent this problem by adapting cytotoxic cells to long term growth with interleukin 2, cloning the effector cells and then testing the target cell specificity of individual clones. Thus we will further examine whether the broad target cell reactivity of poly I-induced effector cells is determined in part by multiple clones of specific effector cells.

3. To more clearly identify the genetic control of broadly reactive anti-tumor cytotoxic cells, we will test selected congenic strains developed by Bailey which carry different complexes of BALB/c genes (low responder) on a C57BL/6 (high responder) background. Moreover, about a 150 additional backcross mice will be tested to define more clearly the ratio of high to low responder mice and to complete our test for linkage of one of the genes to the MHC. CBA and C57BL/6 (both high responder strains) will be used to determine whether there is any MHC restriction of effector cell activity using cultured kidney fibroblasts from appropriate congenic strains as a source of target cells.

4. The relationship of interferon to induction of broadly reactive anti-tumor cytotoxic cells will be analyzed by assaying supernatants of cultures for interferon activity. Preliminary tests with Dr. Kronenberg have demonstrated that interferon activity can be detected in such supernatants. Initially we will simply determine levels of interferon in supernatants from cultures of high and low responder mouse spleen cells and also attempt to block generation of activity with anti-interferon antisera.

Publications

Eggers, A. E., Hibbard, C. A., Civin, C. I. and Wunderlich, J. R.: Chemical enhancement of tumor immunogenicity. J. Immunol. 125:1737-1743, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05016-12 I

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
In Vitro Tests of Immunity in Patients with Melanoma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SUMMARY OF WORK (200 words or less - underline keywords)

Additional data analyses confirm that (1) greater than 90% of melanoma patients receiving melanoma vaccine generated cellular and/or humoral immune responses against one or more of the vaccine cell lines; (2) PBL from melanoma patients who remain in remission (except for those receiving chemotherapy) had a normal capacity for generating cytotoxic cellular immune responses in vitro, and to this extent were not immunosuppressed; (3) treatment of melanoma patients with a nonspecific immunostimulant (BCG) did not increase the average natural killer cell or K cell (ADCC effector) cytotoxic immune activity; and (4) primary in vitro cytotoxic cell responses against vaccine cell lines were impeded a few months before clinically detectable relapse.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The objectives of this project have been to carry out in vitro tests for cytotoxic humoral and cellular immunity to determine 1) if patients respond immunologically to the melanoma vaccine and 2) if immunologic responsiveness correlates with disease progression. Patients being tested are associated with an NCI melanoma immunotherapy protocol.

Methods Employed: 1) Direct, Cell-Mediated Cytotoxicity: Cytotoxicity is measured by the release of ^{51}Cr from labelled target cells incubated overnight with immune cells. Target cells are prepared from vaccine melanoma cell lines maintained in tissue culture. Normal and immune lymphoid attacking cells are prepared from human peripheral blood. Peripheral blood cells are purified by centrifugation with Ficoll-Hypaque. Studies of multiply transfused humans have shown that cytotoxicity in this assay is specific for direct cell-mediated reactions and independent of conventional complement, humor antibody, and nonspecific toxins.

2) Lymphocyte Dependent Antibody (LDA) Mediated Cytotoxicity. This cytotoxic pathway is also measured by a chromium release assay. After ^{51}Cr labelling melanoma target cells are washed by centrifugation and then incubated for 1/2 hour with heat-inactivated serum or plasma containing LDA. Target cells are again washed and then incubated with nonsensitized attacking lymphoid cells from normal donors for 4 hours.

3) K Cell Activity. This test is basically carried out as an LDA mediated cytotoxicity assay. Known LDA-containing, poly specific antisera from multi-transfused donors are used as sources of LDA, and peripheral blood lymphoid cells from melanoma patients are used as effector cells. By titering the dose of effector cells, K cell activity (i.e., which will elicit LDA mediated cytotoxicity) from patients' cells can be compared with the counterpart from normal donors' cells.

4) Complement-Dependent, Antibody-Mediated Cytotoxicity. The complement assay is a two-stage, ^{51}Cr-microcytotoxicity test utilizing rabbit complement and vaccine melanoma cell lines.

5) Natural Killer Cell Activity. Cytotoxicity is measured against an allogeneic ^{51}Cr labelled target cell, K562, known to be a sensitive indicator for natural killer cell activity. The assay runs 6 hours and utilizes Ficoll-Hypaque purified peripheral blood lymphocytes.

6) Cytotoxic Cellular Memory. Ficoll-Hypaque purified peripheral blood lymphoid cells are mixed with x-irradiated or mitomycin C treated allogeneic cells in medium with AB positive human serum. After 3 days co-incubation in vitro, cells are harvested, adjusted to a known viable cell concentration, and tested for cytotoxic activity against ^{51}Cr-labelled target cells in an over night direct cell-mediated cytotoxicity assay (see above). As in all of the cell-mediated assays, panels of target cells are used to assess specificity of the cytotoxic reactions.

Major Findings: The following findings were established prior to September 1979 and are reiterated here. The project is being held "open" until final manuscripts are in press. As of December 1978, 130 melanoma patients were tested for humoral and cellular activity against one or more of the vaccine cell lines. Thirty of thirty-five patients who received the melanoma vaccine showed significant cellular immune cytotoxic activity against at least one of the vaccine cell lines. Only two of these patients had significant activity before initiation of therapy. The time course of the cytotoxic cell response was unique for each individual beginning as soon as two weeks after initiation of therapy and continuing in an oscillating pattern in many patients throughout therapy. Since levels of activity oscillated, with no significant activity at times, identification of a "responding" patient was facilitated by use of higher order statistics for analyzing peak levels of activity rather than simply averaging all levels of activity. Previously, we had noted declines in activity associated with disease relapse in the patients receiving vaccine. Multifactorial analysis reveals that these declines are not significantly associated with disease progression; other variables can account for the reduced activity. The magnitude of the cytotoxic cell response correlated with one technical aspect of the assay, namely the time lapsed between drawing of the blood specimen and the isolation of leukocytes. The activity decreased with the lapsed time. Considering the group of 100 patients on the protocol who did not receive the melanoma vaccine, there was no stimulating effect of other forms of therapy (BCG, chemotherapy, or surgery alone) on cellular immune cytotoxicity. Cytotoxic cellular memory, as evidenced by in vitro antigen-stimulated development of cytotoxic activity against vaccine cell lines, was also evaluated. Half of approximately 30 patients receiving vaccine therapy showed memory activity at some stage of treatment. Such memory activity was undetectable in each of 80 patients not receiving vaccine. Tests for the ability of PBL to generate primary cellular immune responses in vitro have demonstrated that the average response of cells from patients tested within a few months of the time of relapse (approximately 40 patients) appears to be lower than the average response of cells from normal donors and patients who remain in remission. An exception is those patients receiving chemotherapy, approximately 25, whose average response is clearly suppressed.

Significance to Biomedical Research and the Program of the Institute: In vitro cytotoxicity tests are being used to assess the effectiveness of immunotherapy for melanoma patients. These tests help determine 1) if patients are immunosuppressed; 2) if the level of immunosuppression correlates with disease progression; 3) if a vaccine preparation is defective as indicated by failure to stimulate responses in any patient; and 4) if the vaccine has provided non-specific immunostimulation.

Proposed Course of the Project: Publications for this project will be incorporated under other projects by this investigator and this project will be terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05018-11 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Membrane Damage by Immune Mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.A. Henkart	Senior Investigator	I NCI
Other:	M. Oliver	Clinical Associate	I NCI
	J. Todd Lewis	Post-doctoral fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.8	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Three different aspects of this project are currently underway: (1) Studies of membrane damage by complement using resealed erythrocyte ghosts have concentrated on possible explanations for the heterogeneity of functional membrane pore sizes. Increasing complement dose allows efflux of larger markers from ghosts; varying the C8/C9 ratio by using a reactive lysis system in which individual components are added suggests variations in this ratio may explain some of heterogeneity. (2) Large unilamellar liposomes were made with radioactive markers trapped inside, and a technique based on immunoprecipitation developed to separate liposomes from released marker. Sieving by low detergent concentrations, seen previously in ghosts, was confirmed in this system. (3) To test the hypothesis that cytotoxic T lymphocytes lyse their target cells by causing membrane damage, techniques for creating planar lipid bilayer membranes containing H-2 antigens are being developed. H-2 antigens were purified by affinity chromatography using hybridoma anti-H-2 antibodies and reinserted into liposomes.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Our overall objective is to define the mechanisms by which lymphocytes destroy foreign cells. To this end we have dissected the killing process into discrete steps which can be studied independently. In this project we have concentrated on the membrane lesion induced by lymphocytes in the target cell. Based on previous work by us and others, we hypothesize that killer lymphocytes implant molecules into the target cell membrane which injure its permeability properties. This is analogous to the mechanism of complement action, and other agents are known to act in this way; we are studying such agents to compare their actions to lymphocyte damage. This project seeks experimental support for the above hypothesis of lymphocyte mediated target membrane damage.

Methods Employed: (1)Erythrocyte ghosts were prepared with two different radioactive markers of different size resealed inside. Their release was followed by pelleting the ghosts by centrifugation and counting the supernatent. Complement was activated by using antibody, or by using a "reactive lysis" system in which C5b-6 was mixed with C7, and then subsequently with functionally purified C8 and C9. (2)Large unilamellar liposomes made of dioleoyl phosphatidyl choline and 1% NBD-PE were made by octylglucoside dialysis. A mixture of ^{51}Cr-EDTA and ^{125}I-protein was trapped inside the liposomes during their formation. The liposomes were separated from the released marker by adding anti-TNP (which cross-reacts with NBD) and then fixed Staph. aureus bearing protein A, incubating, and spinning down the bacteria and bound liposomes. The supernatent was counted to measure marker release. (3) H-2 K^k antigen was purified from RDM-4 lymphoma cells by detergent solubilization, lentil lectin and 11-4.1 hybridoma affinity columns. SDS gels show the major band is at 45,000 molecular weight. The H-2 antigen was reinserted into liposomes by sodium deoxycholate dialysis. Very large liposomes were grown by swelling dried lipid at low ionic strength, and purified by centrifugation. These large liposomes were trapped in a small oriface in Saran mounted in a specially designed chamber, so that the electrical properties of the membrane could by monitored.

Major findings: (1) Using antibody-coated ghosts and whole guinea pig complement, small markers such as sucrose are released at much lower complement concentrations than protein markers of 15,000 molecular weight; such protein markers of less than 40,000 molecular weight are released from ghosts by complement as observed by us and others previously. Some experiments have shown that increasing the C9/C8 ratio gives sieving properties indicative of larger pores using small markers such as inositol, sucrose, and raffinose. This would indicate that stoichiometry of the proteins in the C5b-9 complex could explain some or all of the observed pore size heterogeneity. (2) Immunoprecipitation techniques can be used to separate liposomes from released marker. This represents a major technical advance over previously used column techniques, which are not convenient for performing a large series of experiments. Using this method, we found that very small concentrations of the detergents Triton X-100 and sodium deoxycholate create apparent pores in liposomes; ghosts treated under these conditions were previously found to show similar sieving properties to complement treated ghosts, leading to speculation that

there may be similar molecular mechanisms in both situations. (3) Although no experiments with cytotoxic T lymphocyte interactions with planar lipid bilayers have yet been performed, steady progress has been made in developing this technically difficult system.

Significance to Biomedical Research and the Program of the Institute: The process of lymphocyte destruction of foreign cells may be one of the most important mechanisms for the immunological rejection of allografts and tumors in vivo. Studies such as ours, directed at the cellular and molecular nature of this process, allow a more complete understanding of the basic knowledge of the body's immunological defense system against foreign cells, including malignant cells.

Proposed Course of Project: We will extend the kinds of studies we have previously carried out on ghosts to liposomes, which have the advantage that their lipid composition can be varied. A variety of membrane-active agents will be tested to see what kind of permeability characteristics they induce. Continued attempts are being carried out as indicated to demonstrate membrane damage on artificial membranes by cytotoxic T lymphocytes as well as other types of cytotoxic lymphocytes.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05021-10 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Antigens Determined by the Murine Major Histocompatibility Locus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	H. Auchincloss	Clinical Associate	I NCI
	J. A. Bluestone	Postdoctoral Fellow	I NCI
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COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION Transplantation Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	3.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies are being directed toward understanding the major histocompatibility complex, the structure and function of the products of this complex, and manipulations of immune responses to these products. Current studies include: 1) Characterization of major histocompatibility antigens: Congenic resistant strains of mice are developed, maintained, and used in serologic and immunochemical analyses of the MHC products of the mouse; 2) Studies of monoclonal antibodies to H-2 and Ia antigens: Hybridoma cell lines are produced by fusion of immune mouse spleen cells with mouse myeloma cells. The monoclonal anti-H-2 and anti-Ia antibodies produced by these hybridomas are analyzed by serologic and immunochemical means and are used to further characterize the fine structure of the MHC; 3) Characterization of receptor sites for histocompatibility antigens: Anti-idiotypic antisera are being produced against anti-H-2 and anti-Ia hybridoma antibodies, and the effects of these antisera on in vitro and in vivo parameters of histocompatibility are being assessed; and 4) Mechanism of tolerance to H-2 and Ia antigens: The humoral and cellular responses of radiation bone marrow chimeras are being examined, and the mechanism for maintenance of tolerance in these animals is being studied.

Project Description

Objectives: 1) To produce antibodies against the products of defined regions of the major histocompatibility complex. 2) To characterize the reactions of these antibodies with the cell surface of lymphocytes, and to determine the nature of the cells bearing individual antigens. 3) To characterize the products with which these antibodies react by immunochemical means. 4) To attempt to produce anti-idiotypic antibodies against the receptors on these antibodies which detect cell surface histocompatibility antigens.

Methods Employed: 1) Congenic resistant strains of mice differing only at their major histocompatibility loci have been reciprocally immunized in order to produce antibodies of known, restricted specificity. The antibodies have been characterized by assays of complement-mediated lymphocytotoxicity and by fluorescence microscopy using a fluoresceinated rabbit antimouse immunoglobulin as a developing agent.

2) Hybridoma cell lines are obtained by fusion of immune mouse splenic lymphocytes with mouse myeloma cells (SP2/0, P3U1, and NS1). The cells are mixed and exposed to polyethylene glycol, 30% for 8 minutes, and fused cells are then cultured in the presence of a selective medium (HAT) for 2 weeks in microtiter wells. Hybridoma cells secreting anti-MHC antibodies are detected by a complement-mediated cytotoxicity assay on individual microwell supernatants. Positive cultures are then sequentially cloned in vitro and in some cases passed in vivo to produce large amounts of ascites hybridoma antibodies.

3) Cell surface antigens reactive with anti-H-2 and anti-Ia antibodies and monoclonal antibodies are isolated and studied immunochemically. Cells are labeled in vitro with ^{3}H-leucine, solubilized in a nonionic detergent, purified by lentil lectin chromatography, and then mixed with the antibodies being analyzed. Complexes are precipitated with Staphylococcus aureus Cowan I strain which contains protein A on its surface, and the precipitated complexes are then dissociated in SDS and mercaptoethanol and analyzed by polyacrylamide gel electrophoresis.

4) Hybridoma antibodies against H-2 and Ia antigens are purified by affinity chromatography on protein A Sepharose columns and are then used to immunize heterologous animals. Pig, rabbit, and goat antihybridoma reagents have been produced. These reagents are absorbed exhaustively on normal immunoglobulins or myeloma proteins in order to remove anti-isotype and antiallotype antibodies, and the putative anti-idiotypic antibodies are then absorbed and eluted from the relevant hybridoma antibody. These anti-idiotypes are then analyzed by hemagglutination and hemagglutination inhibition assays using cells to which the immunizing hybridoma, different hybridomas, or normal immunoglobulins have been coupled.

5) Cell-mediated cytotoxicity assays are carried out using mouse splenic lymphocytes as both responders and stimulators. The effects of antibodies to cell surface antigens at both the target and killer cell level are analyzed in the 4 hour cytotoxicity assay.

6) Mice are treated in vivo with purified anti-idiotype antibodies. The effect of such treatment on idiotype levels and anti-H-2 antibody activity in the serum of these animals is examined. In addition, spleens from these animals are examined for in vitro MLC and CML reactivity. Treated animals are also examined for in vivo reactivity to appropriate skin grafts, and humoral and cellular immunity following grafting are examined by complement-mediated cytotoxicity assays and by CML assays.

Major Findings: 1) Antibody responses to at least 2 H-2 public specificities have been shown to be genetically controlled by non-H-2-linked genes.

2) Crossreactions have been detected between the Ia antigens of mice and those of several other species, including rat, pig, and human being.

3) Fusion of spleen cells from mice hyperimmunized against H-2 antigens and boosted 2-3 days before fusion has been found to give satisfactory results in the production of anti-H-2 hybridomas. About 50 stable hybridomas have so far been produced, most of which detect H-2 or Ia antigens of a variety of haplotypes. Panel testing has indicated that most of these antibodies react with public specificities of the H-2 and Ia antigens, while a few appear to detect private specificities. Numerous crossreactions have been detected using these monoclonal antibodies, which define a variety of new public H-2 and Ia specificities.

4) Analysis of anti-H-2 antisera and monoclonal antibodies by gel electrophoresis of labeled cell surface antigens has demonstrated two new H-2 products determined by loci within the D region, named H-2L amd H-2R. Three hybridoma antibodies reactive with H-2L and/or H-2R antigens have been produced. These antibodies are being used for structural studies of the H-2L molecule.

5) Antibodies directed against the killer cell have been shown to block cell-mediated cytotoxicity against the H-2 products. The specificity of these antisera has been shown to be directed to a product of a gene closely linked or identical to the Ly-2 locus.

6) Anti-idiotypic antibodies reactive with three of the hybridoma anti-H-2 antibodies and with two of the hybridoma anti-Ia antibodies have been produced and have been shown to be specific by hemagglutination inhibition assays. Assessment of a variety of hybridoma antibodies and immune sera for the presence of these idiotypic specificities has indicated that at least one of the anti-Ia idiotypes is prevelant in the normal immune response.

7) The effect of monoclonal anti-H-2 antibodies on the effector phase of CML reactions has been examined. All anti-H-2K^k monoclonal antibodies tested were found to block CML effectively, confirming that serologic specificities are on the same molecules which are detected by CML reactions.

8) Treatment of animals with anti-idiotypic antibodies has been found to induce appearance of idiotype in the serum of these animals. This has been true for all of the anti-H-2 and anti-Ia anti-idiotypes so far examined. In

addition, a percentage of the induced idiotype has been shown to bear the same anti-H-2 or anti-Ia specificity as the original monoclonal antibody. These findings therefore represent the induction of anti-H-2 and anti-Ia antibody responses in the absence of exposure to the actual antigens.

Significance to Biomedical Research and the Program of the Institute: 1) The H-2 and Ia antigens are cell surface determinants which appear to be involved in physiologic cell-cell interactions in the immune response. Therefore, antisera against these antigens provide tools for dissecting the mechanism of these cell interactions and possibly for modifying responses.

2) The specificity of the receptor for histocompatibility antigens should reside in the variable portion of the heavy and light chains of the relevant antibody molecules. Thus, anti-idiotypic antibodies against such receptors might be expected to distinguish those cells capable of reacting against individual histocompatibility antigens. Such antibodies thus provide an approach to modification of the immune response to cell surface antigens. Our findings on induction of idiotype by in vivo treatment with anti-idiotype indeed indicate that such modifications are possible.

Proposed Course of Project: 1) In order to maintain isogenicity of the background of our congenic lines, backcrosses of these lines to the reference congenic partner will be performed at least once every ten generations. Backcrosses of congenic lines will be examined for further recombination events within the H-2 and I regions. Recombinants will then be examined for fine structure analysis of the MHC and for the production of antisera against new H-2 and Ia specificities.

2) Anti-idiotypic antibodies directed against anti-H-2 and anti-Ia receptors will continue to be produced and studied. These reagents will be examined for reactions with antibodies produced in conventional immunization schemes in order to determine the prevalence of the individual combining sites.

3) The effects of anti-H-2 and anti-Ia anti-idiotypic reagents on MLC and CML reactions will be examined, in order to detect possible sharing of idiotypes between T and B cell receptors.

4) Strain distribution studies and immunoprecipitation analyses will be used in order to determine the extent of heterogeneity of the newly defined H-2L locus.

5) Further fusions of immunized cells from a variety of different strain combinations will be performed in order to produce additional monoclonal hybridoma antibodies to a variety of H-2 and Ia specificities. The library thus obtained will be screened by serologic and immunochemical means in order to further characterize the products of the MHC.

6) The effect of in vivo treatment with anti-idiotype antibodies will continue to be examined. The effects of such treatment on skin graft rejection and on humoral antibody production and CML reactivity will be studied. The possible

sharing of idiotypes between alloantigen receptors and modified self receptors will also be examined.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05023-10 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Transplantation Antigens of Swine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	J.-J. Metzger	Expert	I NCI
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COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A breeding program has been carried out starting with two miniature pigs from different sources and selecting offspring according to tissue typing procedures aimed at defining the major histocompatibility complex of this species. By this procedure three herds of miniature swine, each homozygous for a different set of histocompatibility antigens at the MHC have been developed. Current projects include: 1) Assessment of survival of organs and tissue transplants among and between members of these herds as a model for tissue typing and transplantation; 2) Purification and characterization of the major histocompatibility antigens of this species, and isolation and characterization of peptides from these antigens for sequence analyses and for assessment of immunologic reactivity; 3) Assessment of the immunologic parameters involved in tolerance to allografts in this species; and 4) Detection and characterization of intra-MHC recombinants. Two intra-MHC recombinants have been obtained and are being bred to homozygosity. These should permit assessment of the effects of matching at different MHC regions on the outcome of transplantation in this animal model.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To develop and maintain three strains of miniature swine, each homozygous for a different set of histocompatibility antigens at the major histocompatibility locus (MSLA). The animals are used for in vivo experiments in organ and tissue transplantation and as a source of large numbers of cells from which cell surface antigens are isolated, purified, and characterized chemically.

Methods Employed: Unlike classical inbreeding schemes which require about twenty generations to produce homozygosity, the approach being used with these swine involves selective breeding on the basis of histocompatibility typing of parents and offspring.

Four males and four females were purchased from commercial sources, and mating pairs were selected to be as varied as possible in order to assure the selection of different histocompatibility genes. Skin grafts were exchanged between the members of each pair and sera were obtained from the animals two weeks after the rejection of the grafts. The sera were tested for cytotoxic antibodies by lymphocytotoxic typing, and pairs which produced strong cytotoxic antibodies were bred.

Offspring from each breeding were tested serologically to determine which histocompatibility antigens had been inherited from the parents, and offspring which could be shown to possess the same antigen combinations were selected for further breeding. This process has now been repeated for six consecutive generations.

Transplantation of tissues in these animals has been performed in the large animal facility of the Surgery Branch in Building 14. Methods have been developed for the transplantation of split thickness skin grafts from the ear to the dorsal thorax. Allografts are always placed side by side with autografts as a control. The use of ear skin permits very accurate assessment of viability of the grafts and determination of rejection times. Surgical techniques for vascularized grafts (kidney and liver) are being developed.

Chemical purification studies on swine transplantation antigens are being performed using both detergent and papain solubilized cell surface preparations from spleen and lymph node cells. Initial studies involved radiolabelling of the surface antigens with tritiated leucine by published methods, and the assessment of molecular weight of those components which react with alloantisera by the use of SDS polyacrylamide gels.

In order to produce large quantities of histocompatibility antigens without the use of radiolabeled amino acids, membranes have been prepared from lymphoid organs and solubilized with detergents or by limited papain digestion. The extracts were then purified by lentil lectin chromatography and by affinity chromatography on anti-β2 microglobulin columns, or, in the case of papain solubilized antigen, by DEAE ion exchange chromatography and by gel filtration. Sera produced between outbred swine which react with public

specificities shared in the inbred minipig population are being used to separate products of different histocompatibility loci. Amino acid sequencing of heavy and light chains from purified SLA antigens is being performed. Methods are also being developed for the preparation and isolation of peptides from these isolated antigens.

Major Findings: 1) Breeding of further generations has continued to be successful. Typing of the fourth generation offspring yielded three breeding pairs of animals homozygous for serologically determined transplantation antigens. We therefore now have the capacity to breed three different herds of swine, each homozygous for a different MSLA antigen.

2) Skin graft survival has been found to be prolonged within each of the homozygous herds. The mean survival time for grafts within homozygous herds was 11.8±.89 days, while the mean survival time of skin grafts between animals of the three different herds was 7.0±.36 days.

3) Renal allografts within the DD homozygous herd appeared to survive indefinitely despite minor histocompatibility antigen differences. Rejection occurred following allografts within the other two herds, with variable kidney survival times.

4) Skin grafts to DD recipients maintaining a DD allografted kidney showed marked prolongation of survival. DD kidneys transplanted after skin graft rejection by DD recipients were rejected in hyperacute fashion, but no antibody was detectable.

5) Alloantisera between the three herds have been analyzed by gel electrophoresis using detergent solubilized cell surface antigen preparations. Peaks were obtained at 45,000 molecular weight corresponding to the mouse H-2 antigen analog. Peaks at 35,000 and 28,000 molecular weights corresponding to mouse Ia antigens were also observed. Both by size criteria and by genetic criteria these antigens thus appear to be the precise homologs of H-2 and Ia antigens of the mouse.

6) Milligram quantities of unlabeled histocompatibility antigens have been prepared from individual pig spleens by lentil lectin chromatography and anti-$\beta 2$ microglobulin affinity chromatography. The material eluted from these columns has been shown to consist predominantly of 42,000 and 11,000 dalton molecules and has been assessed immunologically by its ability to inhibit complement-mediated lysis of pig cells by anti-MHC antisera.

7) N-terminal amino acid sequences have been obtained for SLA antigens isolated from two of our three partially inbred lines. Comparisons of these sequences with each other and with sequences of MHC antigens from other species reveal high levels of homology, as well as possible allotypic differences.

8) Two recombinants within the MHC were detected by screening of the progeny of MHC heterozygous animals using MLC reactivity and the cytotoxic assay as

markers. Both new recombinants involved separation of the MLC stimulatory locus (SLA-D) from the serologic loci (SLD-A,B,C). SDS-PAGE anlayses of cell surface antigens from these animals have indicated that the Ia antigens segregated with the MLC stimulating determinants in both recombination events, confirming the identity or close linkage of the genes responsible for both of these products in this species. These recombinants have also been used to produce large amounts of antisera specific for Ia or SD antigens of this species, which were not previously available.

Significance to Biomedical Research and the Program of the Institute: One of the major problems in the study of cell surface antigens of human beings is lack of control of genetic constitution. The use of mice and rats as experimental models avoids this problem, but creates two new ones: 1) physiologically and anatomically these animals are often so different from human beings as to make comparisons and applications of findings difficult. 2) The size of these rodents makes it extremely difficult to obtain sufficient cells and tissues to permit quantitative chemical characterization of relevant cell surface antigens.

For both of these reasons it is desirable to have animals of size comparable to human beings, whose genetic constitution with respect to histocompatibility can be controlled. The miniature pig, which attains an adult weight of about 200 lbs, is ideal for this purpose, and to date the experimental breeding pattern outlined above appears to be working well.

The availability of large quantities of MHC antigens will make it possible to determine the effects of soluble antigen and possibly of peptides from these antigens on tissue transplantation. This will be assessed both by in vitro assays (MLC and CML), as well as in vivo in the transplantation models which have now been developed in these swine. In addition, large amounts of soluble SLA antigens should permit both primary and secondary structural studies to be performed.

Proposed Course of Project: The breeding plan and typing will be continued. Lines will be established and separated, so that herds of any required size in each line can be produced.

Experiments in collaboration with the Surgery Branch will be continued in order to: 1) characterize the immune response to transplantation of skin, kidney, and liver in pigs across defined histocompatibility differences, and 2) to determine the basis of allograft tolerance which has previously been reported in pigs.

Studies of the MSLA antigens at the biochemical level will be continued. Using the isolated unlabeled histocompatibility antigens, we will begin to prepare peptide fragments of these antigens and to analyze these fragments for immunologic reactivities. An attempt will be made to localize those determinants involved in serologic assays (by inhibition of complement-mediated lysis), as well as in cellular assays (CML and MLC). The antigens will also be subjected to further sequence analysis in an attempt to determine

the structural basis for antigenicity in this system. An attempt to crystallize the SLA antigens for x-ray crystallographic studies will be made in collaboration with Drs. Gary Gilliland (Laboratory of Immunogenetics, NIAID) and David Davies (Laboratory of Molecular Biology, NIAMDD).

Publications

Johnson, H., Lunney, J. K., Sachs, D. H., and Flye, M. W.: Preparation and characterization of an antiserum specific for T cells of pigs. Transplantation 29: 477-483, 1980.

Pennington, L. R., Lunney, R. K., and Sachs, D. H.: Transplantation in miniature swine. VIII. Recombination within the MHC of miniature swine. Transplantation 31: 66-71, 1981.

Pennington, L. R., Flye, M. W., Kirkman, R. L., Thistlethwaite, J. R., Jr., Williams, G. M., and Sachs, D. H.: Transplantation in miniature swine. X. Evidence for non-SLA-matched kidney allografts. Transplantation, in press.

Metzger, J.-J., Gilliland, G. L., Lunney, J. K., Osborne, B. A., Rudikoff, S., and Sachs, D. H.: Transplantation in miniature swine. IX. Swine histocompatibility antigens: Isolation and purification of papain solubilized SLA antigens. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05033-10 I

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunotherapy of Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. D. Terry	Director, Immunology Program	IP	NCI
Other:	R. J. Hodes	Chief, Immunotherapy Section	I	NCI
	S. A. Rosenberg	Chief, Surgery Branch	SURG	NCI
	R. I. Fisher	Senior Investigator	M	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A controlled, randomized trial comparing immunotherapy to chemotherapy in stage I and stage II malignant melanoma has been initiated. A total of 181 patients have entered the trial, which is closed to further accrual of patients. Preliminary evaluation of data has demonstrated no significant effect of adjuvant therapies on clinical course.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: This project is designed to study various approaches to the immunotherapy of human malignancies.

Methods Employed: A new protocol (designed IB-2) was initiated in August 1975. This protocol will evaluate the effect of two types of immunotherapy or one type of chemotherapy on the remission duration and survival of patients with stage I (level 4 or 5) and stage II malignant melanoma. Patients are stratified by stage, site of primary, and for stage II patients, presence or absence of clinically palpable lymph nodes and the number of histologically positive lymph nodes. Following stratification, they are randomized to receive no further therapy (this is standard treatment for these patients) or chemotherapy with methyl-CCNU or immunotherapy with BCG, or immunotherapy with BCG plus a vaccine that consists of three tissue culture-grown allogeneic melanoma cell lines. These cell lines were a gift from Dr. Donald Morton, UCLA, and are grown in PPLO-free conditions at Litton Bionetics, Inc., under the supervision of Dr. Edwin Matthews. Cells are treated with the enzyme neuraminidase to remove sialic acid (and thus render them more immunogenic) and frozen until ready for use.

All patients are worked up by the Immunology Branch or the Surgery Branch. Following randomization, they are assigned to the Medicine Branch for chemotherapy, the Immunology Branch for immunotherapy, and the Surgery Branch for follow up if there is no further treatment.

Bloods are drawn during pre-randomization work-up and throughout the treatment cycle for the purpose of serum banking.

Major Findings: As of Dec. 1, 1980, 181 patients have been randomized into this protocol. The state of the trial is summarized in the following table:

	Control	MeCCNU	BCG	BCG & Vaccine
Total Patients Entered	43	46	47	45
Recurrences	29	24	35	24
Deaths	19	20	23	17

In vitro assays have only been carried to the point of indicating that patients are being effectively immunized with the vaccine (see Project No. Z01-CB-05016-10 I). In addition, PPD tests indicate that all patients receiving BCG have converted to a positive skin test.

Significance to Biomedical Research and the Program of the Institute: Immunotherapy studies will explore the clinical effectiveness of manipulating the immune system in patients with cancer, and will also provide new information about the biology of the tumor-host relationship.

Proposed Course of Project: No further patient accrual will occur. For those patients already on study, treatment and follow-up as described by the IB-2 protocol will be continued.

Publications

Terry, W. D., Hodes, R. J., Rosenberg, S. A., Fisher, R. I., Makuch, R., Gordon, H. G., and Fisher, S. G.: Treatment of Stage I and II malignant melanoma with adjuvant immunotherapy or chemotherapy: Preliminary analysis of a prospective randomized trial. In W. D. Terry and S. A. Rosenberg (Eds.), Immunotherapy of Human Cancer. Elsevier: North Holland, New York, in press.

Fisher, R. I., Terry, W. D., Hodes, R. J., Rosenberg, S. A., Makuch, R., Gordon, H. G., and Fisher, S. G.: Adjuvant immunotherapy or chemotherapy for malignant melanoma: Preliminary Report of the National Cancer Institute Randomized Clinical Trial. Surgical Clinics of North American. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05034 09 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Characterization and Isolation of Human Lymphocyte Subpopulations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. B. Dickler Senior Investigator I NCI

COOPERATING UNITS (if any)
none

LAB/BRANCH
Immunolggy Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long term goal is to characterize and isolate human lymphocyte subpopulations and evaluate the function of these populations in the immune response of both normal and diseased individuals. Recent work under this project has shown that anti-HL-A alloantisera contain antibodies against antigens which are found primarily on B lymphocytes and which are associated with B lymphocyte Fc receptors. In addition it was found that the Fc receptors of B lymphocytes and UL cells differ both in their avidity for Ig complexes and in the fact that the Fc receptors of UL cells do not appear to be associated with "Ia-like" antigens. Human peripheral blood mononuclear cells cultured in vitro with the antigen KLH-TNP generated significant numbers (compared to controls without antigen) of plaque-forming cells measured with SRBC coated with staph protein A in the presence of rabbit anti-human Ig, but not when measured with SRBC or SRBC-TNP.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To characterize and isolate human lymphocyte subpopulations and evaluate the function of such populations in the immune response in both normal and diseased individuals.

Methods Employed: Lymphocytes from normal human donors were reacted with alloantisera and then evaluated for the presence of bound antibody and function of Fc receptors by fluorescent techniques. Alloantisera were fractionated by absorption with and elution from platelets. Antigen-antibody complexes and double marker fluorescence techniques were used to evaluate the avidity of lymphocyte subpopulations for Ig complexes. Human peripheral blood mononuclear cells were cultured in vitro with or without soluble protein antigens in order to generate antibody secreting (plaque-forming) cells measured in a plaque-forming cell assay using a variety of indicator cells. Subpopulations of lymphocytes were purified or depleted using a variety of techniques including gradient centrifugation, rosetting, anti-Ig columns, G-10 Sephadex columns, nylon wool columns and adherence. Various markers were employed to evaluate purity of populations including fluorescent detection of surface Ig and Fc receptors, E-rosette formation with SRBC and latex particle ingestion.

Major Findings: Certain anti-HLA alloantisera contained antibodies which inhibited the binding of complexed Ig to B lymphocyte Fc receptors. After absorption with platelets the alloantisera lost HLA reactivity but retained the ability to react with B lymphocytes and inhibit Fc receptor function. Conversely, the HLA antibodies eluted from platelets reacted with all lymphocytes but did not inhibit binding to Fc receptors. The Fc portion of the inhibiting antibodies was not required since $F(ab')_2$ fragments also produced inhibition. These data suggest that human B lymphocytes bear "Ia (immune response gene associated) like" antigens and that these antigens are associated with B lymphocyte Fc receptors. B lymphocytes and UL cells were shown to have Fc receptors which were distinct. UL cells' Fc receptors had a much higher avidity for Ig complexes and were not associated with "Ia like" antigens. These differences may be of functional importance.

Human peripheral blood mononuclear cells from several normal individuals have been cultured in vitro with or without antigen (KLH-TNP). Those cultures with antigen generated significant numbers (compared to controls without antigen) of antibody secreting cells as measured in a plaque-forming cell assay. Plaque forming cells were observed using SRBC coated with staph Protein A in the presence of rabbit anti-human Ig but not with SRBC or SRBC-TNP. A culture period of 6 days, antigen concentration of 10-50 ug/ml, and a cell concentration of 2.5×10^6/ml were found to be optimal.

Significance to Biomedical Research and the Program of the Institute: Characterization and isolation of lymphocyte subpopulations is of fundamental importance for an understanding of the nature of the immune response. By analogy to the murine system, identification of human alloantigens associated with Fc receptors, and primarily expressed on B cells may be important for

understanding immunoregulation and interactions between immunocompetent cells, and may help characterize the genes responsible for such regulation.

Proposed Course of Project: Due to time commitments to other projects, this study was terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05035-09 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Characterization of the Lymphocyte Receptor for IgG

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. B. Dickler	Senior Investigator	I	NCI
Other:	M. L. Lamers	Postdoctoral Fellow	I	NCI
	S. Heckford	Postdoctoral Fellow	I	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	2.25	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to characterize lymphocyte Fc receptors genetically and functionally. Recent findings indicate that the Fc IgG receptors of B lymphocytes interact with: a) the lymphocyte cytoskeleton, b) Ia antigens and Lym antigens, c) surface IgM, and d) surface IgD. Each of these interactions is distinct, specific, and non-random. These findings strongly suggest that B lymphocyte Fc IgG receptors are central to B lymphocyte activation and immunoregulation.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: 1) To evaluate the genotypic and phenotypic expression of the Fc receptor; 2) to evaluate the role of this receptor in the immune response.

Methods Employed: Heat-aggregated immunoglobulin and/or antigen-antibody complexes are fluorochrome or radioactive isotope labelled, and then allowed to interact with isolated lymphocytes or macrophages from animals. Binding is assayed by U.V. and phase microscopy, the Fluorescence Activated Cell Sorter (FACS), or by isotope counting. The interaction is studied by manipulation of the conditions of interaction, and by immunologic and chemical modifications of both lymphocytes and complexes. Genetic studies are carried out utilizing inbred, congenic, and/or recombinant mouse strains. Functionally, the effect of monomeric Ig, antigen-antibody complexes, and ligands specific for molecules which interact with Fc receptors are evaluated using the B lymphocyte responses of proliferation as measured by tritiated-thymidine incorporation, and maturation (antibody secretion) as measured by plaque forming cell assays. The techniques of complement and antibody mediated cytolysis and immunoprecipitation are also employed. A monoclonal antibody specific for Fc receptors has been obtained from Dr. Jay Unkless.

Major Findings: A series of cell surface molecules (Ia antigens) is encoded by genes within the I region of the murine H-2 complex. These are either identical to or closely linked to genes (immune response genes) which regulate immune responsiveness. Anti-Ia antibodies bound to Ia antigens inhibit binding of immune complexes (heat-aggregated IgG or antigen-antibody complexes) to specific cell surface receptors (Fc IgG receptors). The inhibition of specific: (a) the Fc portion of the anti-Ia antibody is not required; (b) binding of ligands to other cell surface molecules (including H-2K, H-2D, IgM, IgD, and lectin receptors) does not produce inhibition; (c) anti-Ia antibodies bound to Ia antigens do not inhibit detection of other surface molecules; and (d) inhibition is observed with some cell types (B lymphocytes and a subpopulation of T lymphocytes) but not others (macrophages and null lymphocytes).

The nature of the Ia antigen-Fc IgG receptor interaction has been examined: (a) these molecules are not identical since binding of antibodies to only a portion of Ia antigens produces maximal inhibition of Fc IgG receptors and the latter can be redistributed without affecting distribution of Ia antigens; (b) ligand-bound Ia antigens do not appear to bind directly to the same site on Fc IgG receptors as immune complexes since occupancy of these receptors by Ig complexes but not ligand-Ia antigens results in an interaction with surface IgM (see below); (c) the interaction does not appear to be mediated by the cytoskeleton since disruption of cytoskeletal function by drugs does not affect the interaction; and (d) the interaction may be steric since monoclonal antibodies against single antigenic determinants on Ia antigens produce only partial inhibition of Fc IgG receptors whereas mixtures of the same monoclonal antibodies produce maximal inhibition; however, immune complexes bound to Fc IgG

receptors do not sterically inhibit detection of Ia antigens. Thus, while the nature of the Ia antigen-Fc IgG receptor interaction has not been fully elucidated, the simplest interpretation is that the two molecules lie in close proximity on the cell surface. A functional role for the interaction is suggested by the non-random and specific nature of the association.

Binding of ligand ($F[ab']_2$ anti-Mu) to surface IgM of B lymphocytes or ligand-induced redistribution of surface IgM has no effect on Fc IgG receptors. However, if the latter receptor is occupied by antigen-antibody complexes which themselves provide insufficient cross-linking to cause redistribution or monomeric IgG at physiologic concentrations then ligand-induced redistribution of IgM results in redistribution of the Fc IgG receptors. The interaction is specific and unidirectional: (a) redistribution of Fc IgG receptors by further cross-linking does not result in redistribution of monomeric ligand occupied IgM; (b) ligand-mediated redistribution of IgM does not result in redistribution of ligand occupied Ia antigens or monomeric ligand occupied IgD; (c) cross reactions between ligands was excluded. A smilar interaction has been demonstrated for surface IgD and Fc IgG receptors except that the interaction only occurs on a subpopulation of B lymphocytes, and only with complexed IgG but not monomeric IgG. These results suggest that whenever IgM is involved in a B lymphocyte response, then the Fc IgG receptor is also involved, and the differences between the sIgM-Fc IgG receptor and sIgD-Fc IgG receptor interactions provide a mechanism whereby the two antigen receptors could provide different signals to the B lymphocyte.

Spleen B lymphocytes can be caused to proliferate by incubation with $F(ab')_2$ anti-mu. If supernatants from spleen cells incubated with the mitogen concanavalin A are included in the incubation the B cells mature into antibody secreting cells. These results confirm those of others and indicate that interaction with surface IgM conveys a signal to the B lymphocyte but other signals are also required to produce maturation. This system is ideally suited to carrying out the functional objectives of this project.

Significance to Biomedical Research and the Program of the Institute: The B lymphocyte Fc IgG receptor appears to play a central role in B cell activation and immunoregulation. Interactions between membrane molecules may be a general mechanism employed by cells to respond to stimuli. The ability to regulate the immune response (possibly via manipulation of Fc IgG receptors) could lead to new forms of therapy for cancer and other diseases.

Proposed Course of Project: 1) Analysis of membrane molecule interactions on different cell types (T lymphocytes, macrophages). 2) Analysis of other cell surface molecules on B lymphocytes for interactions. 3) Analysis of the effects of cell proliferation and differentiation on membrane molecule interactions. 4) Evaluate the effects of monomeric IgG and antigen-antibody complexes in functional assays of B lymphocytes. 5) Evaluate the effects of ligands specific for molecules which interact with

Fc receptors in functional assays of B lymphocytes. 6) Evaluate the effects of monoclonal antibody specific for Fc receptors on B lymphocyte function.

Publications

Dickler, H. B. and Kubicek, M. T.: Interactions between lymphocyte membrane molecules. I. Interaction between B lymphocyte surface IgM and Fc IgG receptors requires ligand occupancy of both receptors. J. Exp. Med. 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05036-09 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic Control of the Immune Response to Staphylococcal Nuclease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. H. Sachs	Chief, Transplantation Biology Section	I NCI
OTHER:	R. Hodes	Chief, Immunotherapy Section	I NCI
	P. Nadler	Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies directed against idiotypic determinants on anti-Staphylococcal nuclease antibodies from different mouse strains have been produced in rats and in pigs. The idiotypes are detected by hemagglutination assays and by the inhibition of antibody-mediated inactivation of nuclease. By screening a variety of strains and offspring from appropriate matings between strains for the presence of idiotypes and other markers, it has been shown that idiotype expression is linked to the heavy chain allotype markers. By means of an in vitro anti-TNP nuclease plaque-forming cell response, idiotypic markers have been demonstrated on T helper cells. Administration of anti-idiotypic antibodies to mice has been found to induce idiotype expression in the serum of these animals. This effect appears to involve T cells, since it is not observed in nude mice, and since idiotype-bearing T helper cells for in vitro anti-TNP nuclease response have been found in spleens from such treated animals. Isoelectric focusing studies on anti-nuclease antibodies have indicated characteristic spectrotypes for different strains which appear to correlate with idiotype expression.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Staphylococcal nuclease (nuclease) is a protein enzyme, the immune response to which we have found to be under genetic control by H-2-linked Ir genes in the mouse. Because of the wealth of available data on structural and immunochemical properties of nuclease, it is an ideal model antigen for use in dissecting the mechanism of genetic control of immune responses. Such mechanistic studies at both the T cell and B cell levels are the objective of this project.

Methods Employed: 1) Antibodies to NASE have been prepared in groups of mice of a variety of strains (both high and low responders) differing in H-2 type or allotype or both. These antibodies have been purified by affinity chromatography. Inbred Lewis rats and miniature swine have been immunized with the purified anti-NASE antibodies obtained from immune ascites from A/J mice, SJL mice, B10.A(2R) mice, and BALB/c mice after immunization with NASE. Immunoabsorptions with normal immunoglobulins from the same strains have been used in order to determine whether or not antibodies reactive with the variable region (i.e., anti-idiotypic antibodies) have been produced.

2) Anti-idiotypic reactions have been quantified by hemagglutination and by the inhibition of antibody-mediated enzyme inactivation. Such reactivities have been screened against anti-NASE antibody populations from the other strains of mice in order to determine the possible genetic linkage of idiotype to allotype and/or H-2 type.

3) Backcross animals have been screened for antibody levels and for allotype and idiotype expression in order to determine linkage of idiotype to other genetic markers.

4) Antibodies to nuclease have been separated on affinity columns into subpopulations directed against different determinants of nuclease. The reactions of anti-idiotypic antisera with these subpopulations have been characterized in order to determine new idiotypic markers.

5) Animals have been injected with purified anti-idiotype antibodies and the effect of this treatment on their subsequent expression of idiotype and anti-nuclease activity has been examined by means of spectrophotometric and hemagglutination assays.

6) Anti-nuclease antisera and purified antinuclease antibodies have been examined by isoelectric focusing, using either protein stains or autoradiography employing ^{125}I-labeled nuclease or purified anti-idiotype.

7) An assay for secondary immune responses to TNP-nuclease in vitro has been developed. This assay has been used to examine the genetics of secondary responses to nuclease, as well as to assess the effects of anti-idiotype on the in vitro response and the cell level of expression of idiotype in this response.

Major Findings: By two separate criteria, anti-idiotype antibodies have been obtained. 1) After exhaustive absorption with whole normal A/J ascites or repeated passages over affinity chromatography columns to which normal A/J ascites was bound, antibodies remained which reacted with A/J anti-NASE but not with B10.A anti-NASE antibody. Prior to absorption these rat antibodies contained precipitating antibodies to either type (A/J or C57BL/10) of antibody but no precipitating antibodies remained following absorption. 2) The rat anti-A/J anti-NASE antibodies prior to and following absorption reacted with the combining site of anti-NASE antibodies as evidenced by their ability to inhibit the anti-NASE antibody-mediated inactivation of NASE in an enzymatic assay.

Among (B10.AxA/J)xB10.A backcross animals the A/J anti-NASE idiotype was found to be linked ($p < .005$) to heavy chain allotype. However, a large recombination frequency was found (7-10 percent). The use of a very sensitive assay for allotype indicated that this recombination frequency was not the result of faulty allotypic typing. Also, progeny testing of the putative recombinant animals showed approximately 50 percent of the offspring to also have recombinant phenotypes, further indicating that these were true recombinant animals.

Injection of pig anti-idiotype antibodies into virgin mice has led to an increase in the level of idiotype in the mouse serum. That the reactive immunoglobulin molecules induced represented true idiotype and not anti-anti-idiotype was indicated by the fact that this induced idiotype was detectable using anti-idiotypic reagents produced in a variety of species. Similar treatment of nude mice did not lead to idiotype expression in the serum, suggesting that the effect may involve T cells. The idiotype detected in serum from the anti-idiotype treated mice was predominantly found on immunoglobulin molecules not detectably specific for nuclease. Treatment of such animals with nuclease led to an even greater increase in idiotype expression, and in this case there was also an increase in idiotype expression on anti-nuclease antibody molecules.

Spleen cells from anti-idiotype primed animals were found to provide T cell help in an in vitro anti-nuclease TNP plaque-forming cell response. This T cell help was equivalent to that obtained from nuclease-primed spleens. Anti-idiotype was found to inhibit this T cell help and in fact could eliminate the help if complement was added.

Isoelectrofocusing studies on anti-nuclease antibodies from a variety of strains have indicated that each strain has a characteristic spectrotype. Development of the isoelectrofocusing gels with ^{125}I-labeled anti-idiotype antibodies has shown a striking similarity in the clonotypes detected in all mice of a given strain, and there appears to be marked correlation with the expression of crossreactive idiotypes as detected by serologic means.

Significance to Biomedical Research and the Program of the Institute: Many of the antigens being studied in this laboratory are difficult to obtain in pure form (such as transplantation antigens and tumor antigens), and it is therefore important to have a well-characterized model protein on which

initial experiments can be performed. In choosing models both for mechanistic studies and for studies in manipulation of the immune response, Staphylococcal nuclease is a particularly attractive protein. The immune response to this antigen has previously been shown in this laboratory to be under genetic control by an H-2-linked Ir gene.

The development of anti-idiotypic reagents directed toward antibodies under the control of an H-2-linked Ir gene provides another handle to study the mechanism of Ir gene function. These antibodies may help to determine whether the specificity of T cell recognition of antigens depends on similar variable region gene products to those responsible for B cell or antibody specificity. They may also lead to possible methods of controlling Ir gene expression. Finally, the detection of idiotypic determinants on T helper cells may provide a means of studying the elusive T cell receptor. Cloned lines of T helper cells bearing idiotype should provide a source for chemical characterization of the putative receptor.

Proposed Course of Project: The mechanism by which in vivo treatment with anti-idiotype leads to increased idiotype production will be pursued. Transfers of purified populations of T cells and B cells from immunized mice and anti-idiotype primed mice into irradiated recipients will be carried out. The effect of administration of inappropriate anti-idiotype antibodies to mice of various strains will be assessed. An examination of the control of idiotype expression at the T cell level will be attempted using the in vitro anti-TNP nuclease plaque-forming cell response.

The isoelectrofocusing patterns on serum from animals treated with anti-idiotype will be performed. These patterns will be compared to those we have previously found following antigen immunization. Attempts will be made to determine the nature of the idiotype expression in anti-idiotype primed animals which do not demonstrate measurable anti-nuclease activity. Such antibodies will be purified by affinity chromatography on anti-idiotype columns and will be examined for spectrotype as well as for their ability to induce anti-idiotype reactive with anti-nuclease antibodies. These studies may help to shed light on the network normally involved in the immune response to nuclease and may permit intervention into that network in a specific manner.

Attempts will be made to produce hybridomas secreting monoclonal anti-nuclease antibodies. These antibodies will then be examined for expression of individual anti-nuclease idiotopes and will be used to produce monospecific anti-idiotope reagents for further characterization in in vitro and in vivo assays. In addition, attempts will be made to produce cloned T cell lines capable of providing T cell help for the in vitro anti-nuclease-TNP response. Such lines will be examined both for anti-nuclease reactivity and reactivity with our anti-idiotypic reagents. Should such lines be established, attempts will be made to isolate and characterize the relevant T cell receptors.

Project No. ZO1-CB-05036-09 I

Publications

Sachs, D. H.: Genetic control of idiotype expression. In Fougereau, M., and Dausset, J. (Eds.): Immunology 80, New York, Academic Press, Inc., 1980, pp. 478-495.

Nadler, P. I., Miller, G. P., Sachs, D. H., and Hodes, R. J.: Genetic control of antibody responses to TNP-nuclease in vitro. J. Immunol., in press.

Sachs, D. H., El-Gamil, M., and Miller, G.: Genetic control of the immune response to staphylococcal nuclease. XI. Effects of in vivo administration of anti-idiotypic antibodies. Eur. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05038-09 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to Hapten Modified Syngeneic Lymphocytes in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
Other:	R. B. Levy	Senior Staff Fellow	I NCI
	H. Fujiwara	Visiting Associate	I NCI
	P. K. Arora	Visiting Fellow	I NCI
	P. A. Henkart	Senior Investigator	I NCI
	T. Tsuchida	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.5	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cytotoxic T lymphocytes (CTL) were generated in vitro against syngeneic spleen cells conjugated with a number of different haptens: trinitrophenyl (TNP-self), two different isomers of fluorescein isoothiocyanate and I-AEDANS (AEDANS self). Genetic control of CTL responses to these haptens was compared. H-$2^{k,a}$ and H-$2^{b,d}$ mouse strains were the respective genetic high and low responders to haptens which conjugate to $-NH_2$ groups. The reverse genetic pattern was observed for CTL responses to hapten-self which conjugate to -SH groups. These differential response patterns raise some interesting possibilities concerning genetic control of immune responses and the self determinants recognized. The role of helper T cells in Ir gene control and hapten specificity was also studied for the above haptens. Ir gene defects were detected at the helper cell level and in CTL precursors or accessory cells. Activation of CTL helpers was found to be specific, but once activated, these helper effects were non-specific.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The primary objectives of this laboratory are to investigate the function of T lymphocytes, the role of self recognition, and the effects of major histocompatibility genes on the murine and human immune systems. These studies are being pursued using mouse and human leukocytes which are sensitized to autologous cells either modified with chemical agents (e.g. the trinitrophenyl group) or infected with viruses (e.g. influenza, cytomegalovirus). The murine TNP-self cytotoxic system serves as a basic model for testing many immunogenetic questions concerning the role of the MHC in immune regulation. The specific objective of this project was to continue in the investigation of immunogenetic parameters associated with the in vitro generation of T-cell mediated immunity of murine cells to syngeneic cells modified with the TNP-group. More explicitly, the project was designed to: (a) analyze the dominance of K^k TNP-self responses over D end TNP-self responses; (b) investigate the possibilities of generating helper T cells in vivo for secondary in vitro cytotoxic responses to TNP-self, and to study the effects of helpers on restriction, Ir gene control, cross-reactivity, CTL specificity, etc.; (e) to compare the in vivo priming effects on different types of T-cell mediated immunity, (e.g., cytotoxicity, delayed hypersensitivity, suppression); and (f) to compare the CTL results observed using TNP-self with those obtained using the FITC-self and AEDANS-self haptens.

Methods Employed: For in vitro generation of CML activity mouse spleen cells were sensitized in vitro to autologous cells conjugated with trinitrobenzene sulfonic acid (TNP-self), N-iodoacetyl-N-(5-sulfonic-napthyl) ethylene diamine (AEDANS-self) or various isomers of the fluorscein hapten, one of which was NH_2-reactive (FITC) and others of which were SH-reactive (5AAF and 6AAF). The effector cells generated were assayed on the appropriate hapten conjugated or untreated ^{51}Cr-labelled target cells.

Helper cells for CML responses were generated by injection of hapten-conjugated syngeneic cells or by skin painting with the hapten. Helper cell activity was determined by co-culture of irradiated spleen cells from immunized mice with those from normal spleen cells.

Major Findings: Helper cell activity for TNP-self was demonstrated in genetic high responder ($H\text{-}2^{k,a}$), but not in low responder ($H\text{-}2^{b,d}$) mouse strains. The activation of helper cells was found to be hapten-specific, but these helper cells (or the factors they generate) were able to help CTL responses for other non-crossreacting (at the effector cell level) haptens when the helper-cells were cultures with the specific hapten plus another hapten. Using this approach, we were able to by-pass the helper cell defect to a particular hapten and demonstrate Ir gene defects at both the helper and CTL precursor or accessory cell levels.

The H-2 linked Ir gene control of CTL responses to hapten-self antigens was found to be independent of the hapten specificity of the CTL, but was found to be associated with whether the modifying agent was reactive with NH_2- or SH- groups. Thus, similar haptens exhibited different Ir gene pattens of response in congenic mouse strains, depending on whether they coupled with

cell surface NH_2- or SH- groups. Modifications of SH- groups was associated with responsiveness in H-2^b but low responsiveness in H-$2^{a,k}$ strains. In contrast, modification of NH_2- groups was associated with high responsiveness in H-$2^{a,k}$ and low responsiveness in H-2^b mice.

Significance to Biomedical Research and the Program of the Institute: The project is of fundamental immunological interest in that it describes a major histocompatibility linked immunological phenomenon involving self recognition. The recognition of self MHC-coded structures in association with foreign antigenic determinants raises the possibility for self recognition as important for autoimmunity and for virally-infected autologous cells. The finding that products of the MHC are important for the antigenic complex recognized as well as for determining immune response potential provides a working hypothesis for bifunctional MHC control of disease susceptibility in those examples of HLA-associated diseases in man. The intricate immunoregulatory phenomena identified in the hapten-self cytotoxic system permits us to look for similar patterns in the virally infected murine and human models, and to attempt to determine whether such mechanisms could be operating in the immune systems of the intact individual, in either an infectious or neoplastic state.

Proposed Course of Project: Investigation of this model will continue in order to determine: (a) whether different self MHC products are recognized by helper, and cytotoxic T cells; (b) whether these Ir genes are specific for the reactive groups; i.e., NH_2- vs. SH- rather than for the haptens themselves; (c) what components of the haptens are recognized as unique and crossreactive determinants by CTL; and (d) the role of accessory (antigen presenting) cells in Ir gene control and CTL specificity.

Publications

Levy, R. B., Gilheany, P. E., and Shearer, G. M.: The role of self and foreign antigenic determinants in allogeneic and self-restricted cytotoxic T-cell recognition. J. Exp. Med., 152: 405-418, 1980.

Fujiwara, H., and Shearer, G. M.: Genetic control of cell-mediated lympholysis to trinitrophenyl (TNP)-modified murine syngeneic cells. I. Expression of Ir gene function at the cytotoxic precursor and helper cell levels in the response to TNP-H-2^b self. J. Immunol. 126: 1047-1051, 1981.

Levy, R. B., Fujiwara, H., and Shearer, G. M.: Helper cell analysis in cytotoxic T lymphocyte responses. I. Activation of specific and cross-reactive helper effects in two hapten-modified self responses. Eur. J. Immunol. 10: 904-907, 1980.

Levy, R. B., Shearer, G. M., Richardson, J. C., and Henkart, P. A.: Cell-mediate lympholytic responses against autologous cells modified with haptenic sulfhydryl reagents. I. Effector cells can recognize two distinct classes of hapten-reactive self sites on cell surface proteins. J. Immunol., in press.

Levy, R. B., Henkart, P. A., and Shearer, G. M.: Cell-mediated lympholytic responses against autologous cell modified with ahptenic sulfhydryl reagents. II. Analysis of the genetic control of cytotoxic responses to sulfhydryl and amino reactive reagents. J. Immunol. in press.

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Fujiwara, H., Levy, R. B., and Shearer, G. M.: Studies on in vivo priming of the trinitrophenyl-reactive cytotoxic effector cell system. III. The effects of priming and the involvement of helper cells in the generation of restricted and non-restricted cytotoxic responses. Eur. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05049-07 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

F_1 Antiparental Cell-Mediated Lympholysis In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: G. M. Shearer Senior Investigator I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse F_1 spleen cells develop cytotoxic activity in vitro against parental cells. The antigenic differences detected appear to be controlled by a structural gene (Hh-1) mapping in or near the D region of the H-2 complex. Although a number of positive correlations have been demonstrated between murine hemopoietic graft rejection in vivo and F_1 anti-parent cytotoxicity in vitro, certain differences have been found between these two F_1 anti-parent reactions. These include the observations that marrow graft rejection does not require priming and is not dependent upon thymus-dependent cells, whereas the development of cytotoxic cells requires sensitization and is effected by T-lymphocytes. The same mechanism is also responsible for the rejection of hemopoietic allografts. Injection of F_1 mice with parental spleen cells (which have graft vs. host potential) results in the specific abrogation of F_1 anti-parent reactions in vitro and in vivo, whereas injection of parental bone marrow is associated with some degree of priming for F_1 anti-parent cytotoxicity.

Project Description

Objectives: The objective of this project was to investigate in more detail the immunogenetic parameters associated with the generation of murine F_1 anti-parent cytotoxic effector cell in vitro, and to compare them with allogeneic cytotoxicity in vitro, and F_1 anti-parent hemopoietic graft rejection in vivo.

Methods Employed: Spleen cells from different F_1 mouse strains were sensitized in culture against parental spleen cells. The effector cells generated were assayed on ^{51}Cr-labelled target cells expressing different H-2 haplotypes.

Major Findings: A number of similarities have been observed between F_1 anti-parent marrow graft rejection in vivo and the development of F_1 anti-parent cytotoxic effector cells in vitro. However, a number of differences were also noted. These include the findings that: a) the in vivo rejection process does not require active immunization, whereas the development of cytotoxic effectors does; b) marrow graft rejection is neither effected by nor dependent upon thymus-derived cells, whereas the F_1 anti-parent effectors and their precursors are T-cells; and c) the recognition responsible for F_1 anti-parent graft rejection appears to involve structures coded for by a gene(s) mapping near H-2D, whereas for F_1 anti-parent cytotoxicity, products of additional genes within the H-2 complex are also important. Similar to allogeneic and TNP-self cytotoxic responses, the generation of F_1 anti-parent effector activity requires the presence of glass-adherent cells.

Since the cytotoxic response to Hh determinants is weaker that that to either alloantigens or TNP-self determinants, attempts were made to enhance the response by injecting F_1 mice with parental and F_1 cells expressing Hh determinants (as a means of priming). Priming effects were obtained with irradiated parental spleen cells or unirradiated parental marrow cells. Unirradiated parental spleen cells resulted in unresponsiveness specific for the haplotype of the parental cells injected.

No work has been done on this project during the past year.

Significance to Biomedical Research and the Program of the Institute: These comparative studies may be of importance for understanding the common and distinct immune pathways for radioresistant and radiosensitive components of graft rejection. Such an understanding may be of significance since marrow graft rejection is considered to be a problem of transplantation.

Proposed Course of Project: Since pre-injection of F_1 mice with parental spleen cells (but not with parental bone marrow cells) results in specific abrogation of parental marrow graft resistance, parental tumor cell resistance, and anti-parental cell cytotoxicity, we plan to investigate the cellular and MHC-linked genetic parameters associated with induction (graft vs. host reaction?) and maintenance (tolerance suppression?) of the induced

F_1 anti-parent unresponsiveness (see accompanying report on the "Effects of graft vs. host reaction on cell-mediated immunity"). We also plan to investigate the possibility that the homozygous state of the parental H-2 gene products provides a significantly greater "dose" of relevant H-2 coded self products that does the heterozygous state of the F_1, by using limiting amounts of known modifying agents (e.g. low concentrations of TNBS) and attempting to artificially create an F_1 anti-parent response. Attempts will be made to provide helper T cells for F_1 anti-parent CTL by helpers for TNP-self and for the H-Y antigen. By using chimeras, studies will be performed to determine whether the F_1 anti-parent CTL response is H-2 restricted.

Due to other more pressing and interesting areas of research, no work had been performed on this project during the past year. It is not certain whether any work will be done on this particular project during the coming year. Since the laboratory is currently involved in investigation of an in vivo correlation of this phenomenon, i.e., effects to graft-versus-host reactions on cell-mediated immunity, Project No. Z01 CB 0588-02 I, any work on the F_1 anti-parental cell mediated lympholysis will be included in that project.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 05050-07 I

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies on the Structure and Function of the Constant Portions of Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. M. Segal	Senior Investigator	I NCI
Other:	J. F. Jones	IPA Investigator	I NCI
	S. K. Dower	Visiting Fellow	I NCI
	C. DeLisi	Physicist	LTB NCI
	R. Siraganian	Head, Section of Clinical Immunology	LMI NIDR
	A. S. Fauci	Head, Clinical Physiol. Sec.	LCI NIAID
	B. F. Haynes	Assoc. Prof. Med.	Duke University

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) Results from detailed equilibrium and kinetic studies conform to a mechanism by which multivalent immune complexes interact with Fc receptors (FcR) on P388D_1 cells. The mechanism emphasizes the role of receptor density in governing the affinity with which a cell binds multivalent ligands. (2) A flow microfluorometric assay has been used to quantitate FcR on subsets of human cells. Tγ cells have a higher receptor density than monocytes, and the 3A1 hybridma antibody distinguishes Tγ cells from other subsets of FcR^+ cells. (3) P388D_1 cells internalize immune complexes rapidly at 37°. FcR-mediated internalization also occurs for IgG-dimers, trimers, and perhaps monomers, but the rate of internalization increases with oligomer size. Subsequent to internalization, most FcR disappear from the cell surface. (4) Theoretical calculations on Clq binding to antibody coated targets predict that binding will be stronly dependent upon antibody density. Free IgG in the medium will inhibit Clq binding but only at low antibody density. (5) Antibody-coated ("franked") human peripheral blood lymphocytes specifically lyse tumor targets. Monocytes are not active in this system. Flow microfluorometric studies showed that antibody remained on the franked effector cell surface for long periods of time after franking.

Project Description

Objectives: (1) To investigate the molecular and cellular bases of the interactions of immunoglobulins with immune effector systems. (2) to study the relationship of antigenic recognition to these interactions; and (3) to find methods by which these interactions can be artifically manipulated with the ultimate goal of increasing immune responses toward pathogens or neoplastic cells and decreasing autoimmune responses.

Methods Employed: Organic synthesis, gel filtration, ion exchange chromatography, polyacrylamide gel electrophoresis, complement fixation, radiolabeling of proteins, tissue culture, binding assays, antibody-dependent cytotoxicity assays, cell separations, flow microfluorometry, computer analyses.

Major Findings: 1. Previously we have developed techniques for measuring bindings of ^{125}I model immune complexes to Fc receptors on cells. Using these methods we were able to quantitate the numbers of Fc receptors on cells and obtain estimates of the affinities of these complexes for the receptors. More recently the accuracy of these measurements has been improved by incubating ^{51}Cr-labeled cells with ^{125}I-oligomers. The numbers of IgG molecules bound per cell were then calculated from $^{125}I/^{51}Cr$ ratios from cells which have sedimented through phthalate oils. The double-label techniques has allowed us to quantitate IgG binding with a precision of 1-2%.

In our original study, the model immune complexes were dimers and trimers of rabbit IgG-anti-Dnp antibodies which had been covalently cross linked at their combining site regions with the bivalent affinity labeling reagent, $(BADL\text{-}pro)_2$-EDA. Since that time, another bivalent affinity label, bis-Dnp-pimelic ester (BDPE) has been synthesized, and oligomers have been prepared from anti-Dnp antibodies cross linked with this reagent. We have now completed equilibrium and kinetic studies of the interactions of these oligomers with FcR, and have defined many of the salient features of the mechanism of binding. The binding of IgG oligomers to Fc receptors can serve as a prototype for many other multivalent immune interactions such as complement binding to antibody-coated target cells (see below), antigen binding to B cells, receptor-ligand interactions when both receptor and ligand are cell surface components, and the binding of antibody to multideterminant antigens.

The simplest model which describes the interaction of an IgG dimer (D) with free Fc receptors (R) on a cell can be written:

$$(1)\quad D + R \underset{}{\overset{K_1}{\rightleftharpoons}} DR \qquad K_1 = \frac{(DR)}{2(D)(R)}$$

where DR is a monovalently bound dimer and K_1 is the equilibrium constant, and

$$(2)\quad DR + R \overset{K_2}{\rightleftharpoons} DR_2 \qquad K_2 = \frac{2(DR_2)}{(DR)(R)} = \frac{(DR_2)}{K_1(D)(R)^2}$$

where DR_2 is dimerically bound dimer and K_2 is the equilibrium constant for the second step. In collaboration with Dr. Charles DeLisi, a mathematical formulation of this model has been developed.

The total bound dimer B_T can be written:

$$(3) \quad B_T = DR + DR_2 = 2K_1 (D)(R) + K_1K_2(D)(R)^2$$

and the total receptors/cells, R_T:

$$(4) \quad R_T = R + 1/2\ DR + DR_2 = R + K_1(D)(R) + K_1K_2(D)(R)^2.$$

Equations 3 and 4 have been used to fit data for the binding of IgG dimers to metabolically-inhibited P388D_1 cells, and similar equations with a third term have been used for the trimer. Values of K_1 have been determined independently in monomer inhibition experiments. Calculations have been made using the MLAB program on the NIH PDP-10 computer.

The binding of BDPE dimers and trimers fit a simple cross-linking model, except that a fraction (approximately one-third) of the oligomers bind with higher affinity than the rest. In contrast monomeric IgG binds with a single affinity, as determined from inhibition studies. The simplest explanation for the high affinity binding of oligomers is that some of the receptors are patched on the cell surface. Since receptor concentrations determine the affinity with which multivalent ligands bind to cells in a simple cross-linking model, receptors at local high concentrations would have a higher affinity for dimers than those in low density areas.

Kinetic studies have been done with oligomers cross-linked with both reagents. The rates of release of bound oligomers (dimers, trimers, and heavier fractions) from cells are accelerated by monomeric IgG. Analysis of this phenomenon for the dimer according to the simple multivalent binding mechanism suggests that (1) the cross linking rate (DR+R --> DR_2) is much greater than the rate at which DR is released from the cell (DR --> D+R) and that the rate of formation of DR is the rate-limiting process in dimer binding. These analyses have yielded rate constants for the formation and release of the monovalently-bound intermediate; neither of these processes is limited by diffusion of dimer to or from the cell surface, nor is the lateral diffusion of receptors in the plane of the membrane limiting in the association reaction.

Binding and release processes have both fast and slow components. The biphasic nature of these kinetics is not predicted by the simple multivalent mechanism, nor is it a result of affinity heterogeneity of ligand binding (none was observed). We suggest that the slow binding and release reactions result from some of the receptors being in environments on cell surfaces which are less accessible to the medium than others.

2. The multivalent binding mechanism has been used to predict how Clq would bind to an antibody-coated target cell in which the antibody is free to diffuse in the membrane plane. Clq is a hexamer and we considered that it would bind, at most, hexamerically to the target cells. We also calculated how free monomeric IgG in the medium would affect Clq binding. Inhibition of binding by free IgG is of interest because Clq in vivo, must bind to antibody-coated target cells in the presence of 10 mg/ml of circulating IgG.

The results of these calculations suggest that target-bound antibody can compete with free IgG for Clq, when the cell-surface concentration of antibody is sufficiently high. Binding of Clq thus does not require a conformational change within the IgG antibody. However when the density of antibody molecules on the target cell is low, Clq will not bind to the target in the presence of IgG. We suggest that circulating IgG inhibit complement-mediated reactions against cells bearing low levels of auto-antibody.

3. We are continuing to study Fc receptors on individual cells by flow microfluorometry (FMF). A technique has been developed for labeling cells with fluorescein or rhodamine such that fluorescence emission is proportional to the number of IgG molecules bound per cell. In addition we can now label cells with two different antibodies and interrogate each cell independently for both labels.

Analysis of human peripheral blood mononuclear cells for FcR shows that monocytes can be differentiated from Tγcells by FcR density alone, the monocytes having a lower average receptor density than Tγ cells. In double label experiments using red FcR reagents and green 3A1 hybridoma antibody, we showed that 3A1 stains only T cells (and not monocytes), and that the Tγcells are stained more brightly than the T non-γ cells by this reagent. These studies show conclusively that monocytes and T γ cells are distinct populations--thus ruling out a recent suggestion in the literature that the Tγ cells may in fact be monocytes.

4. When $P388D_1$ cells are incubated with IgG oligomers at 0°, the oligomers bind to the cells, and remain on the cell surface in equilibrium with unbound oligomer. At 37° however, the oligomers are internalized by the cells, and we have over the past year been examining the internalization process. It has been found that large immune complexes are internalized very rapidly, with a half time of less than five minutes. While on the cell surface, the immunoglobulin remains intact, but after being internalized the IgG is slowly degraded into 50,000 M.W. fragments.

Small non-associating oligomers are also internalized, and internalization can be partially blocked by high concentrations of aggregated IgG. The blockable component is FcR-mediated internalization, while non-blockable internalization most probably is a consequence of fluid-phase pinocytosis. Monomers, dimers, and trimers are all internalized by an FcR-mediated process, and the rate of internalization increases with oligomer size. Internalization reaches a plateau after about 30 min. for reasons as yet unknown.

Bound associating oligomers are internalized rapidly and after internalization, at least 2/3 of the FcR disappear from the cell surface. Presumably the FcR are rapidly internalized with the oligomers, and reexpression of FcR is slow.

5. We have continued the study of "franked" effector cells. Franking procedures cause IgG antibodies to adhere to various types of cells, and in some cases, cause these cells to become potent in vitro killers of antigen-coated target cells. The previous studies were performed with $P388D_1$ cells franked with anti-TNP antibodies as effectors and TNP-coated chicken red blood cells (TNP-CRBC) as targets.

More recently we have observed that franking can occur in other systems. For example, both mouse and rat spleen cells and human peripheral blood mononuclear cells can be franked with anti-TNP antibodies, and will subsequently lyse TNP-CRBC in vitro. In addition, franked rat spleen cells and franked human leukocytes will lyse cells from the human B-cell line, SL, which have been treated with TNBS.

Only some subpopulations of franked human leukocytes will lyse tumor targets; these include both polymorphonuclear cells and lymphocytes. Franked monocytes are not cytotoxic for tumor cells. We have also shown by FMF studies that both the lymphocytes and polymorphonuclear cells retain antibody on their surfaces after franking.

Significance to Biomedical Research and the Program of the Institute: The recognition of foreign substances by antibodies elicits a number of reactions which normally lead to their elimination from the body. The purpose of this project is to examine the molecular events which occur as a result of antigenic recognition. It is hoped that these studies will enhance our understanding of these processes and allow us to better control immune reactions. An ultimate goal is to enhance the immune response toward neoplastic cells.

Proposed Course of Project:

1. We will continue to study internalization of immune complexes and IgG oligomers by $P388D_1$ cells and by normal cells using radioactive measurements and FMF. Specifically, we wish to know whether there is a constant turnover of receptors or whether internalization is signalled by the binding of immune complexes on the cell surface. We would also like to know which cellular structures, e.g. microfilaments, coated pits, microtubules etc. are involved in internalization and which metabolic processes are related to this effector function. In other cells we want to know what happens to cell bound antigen-antibody complexes (e.g., are they internalized or do they remain on the cell surface). All of these questions are fundamental to an understanding of immune processes, and we suspect studies in these areas will require several years.

2. We will continue to use FMF techniques to examine FcR in heterogeneous cell samples. We first want to identify (using double label studies) which subsets of cells bear receptors, and how many. Next we will examine what happens to receptors and bound complexes on cells treated in various ways and to correlate these with functional properties where this is possible.

3. We will be doing experiments to investigate the role of circulating IgG in modulating IgG-mediated effector functions. Such studies will be done with both complement and ADCC effector cells.

Publications

Jones, J. F., and Segal, D. M.: Antibody-dependent, cell-mediated cytolysis (ADCC) using antibody-coated effectors: New methods for enhancing antibody-binding and cytolysis. J. Immunol. 125: 926-933, 1980.

Segal, D. M., Dower, S. K., Jones, J. F., and Titus, J. A.: The role of antibody multivalency in immune effector processes. In Progress in Clinical and Biological Research, Vol. 40 "Biological Recognition and Assembly" David S. Eisenberg, James A. Lake and C. Fred Fox (Eds.). Alan R. Liss, Inc., New York, 1980, pp. 319-326.

Titus, J. A., Sharrow, S. O., Connolly, J. M., and Segal, D. M.: Fc (IgG) receptor distributions in homogeneous and heterogeneous cell populations by flow microfluorometry. Proc. Natl. Acad. Sci. USA 78: 519-523, 1981.

Segal, D. M., Sharrow, S. O., Jones, J. F., and Siraganian, R. P.: Fc(IgG) receptors on rat basophilic leukemia cells. J. Immunol. 126: 138-145, 1981.

Jones, J. F., Titus, J. A., and Segal, D. M.: Antibody-dependent, cell-mediated cytolysis (ADCC) with antibody-coated effectors: Rat and human effectors versus tumor targets. J. Immunol. in press.

Dower, S. K., and Segal, D. M.: C1q binding to antibody-coated cells: Predictions from a simple multivalent binding model. Mol. Immunol, in press.

Segal, D. M., Dower, S. K., Titus, J. A., and Jones, J. F.: Requirements for multivalent interactions between antibody and Fc receptors in binding and lysis during ADCC. In Macrophage Mediated ADCC, Hillel S. Koren (Ed.), in press.

Jones, J. F., and Segal, D. M.: Antibody-dependent cell-mediated cytolysis (ADCC) using franked effector cells. In Macrophage Mediated ADCC, Hillel S. Koren (Ed.), in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05055-06 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Regulatory Influences of Cell-Mediated Immune Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R.J. Hodes Chief, Immunotherapy Section I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION
Immunotherapy Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous investigations have described both antigen-specific and antigen-nonspecific T cell-mediated regulatory mechanisms capable of suppressing the in vitro generation of cell-mediated cytotoxicity. Further experiments have been directed at determining the cellular interactions involved in the in vitro generation of suppressor cells, and at analyzing genetic differences in the abilities of different inbred mouse strains to generate suppressor cells. Most recently, monoclonal anti-Lyt reagents have been employed to characterize the T cells responding in cell-mediated cytotoxicity as Lyt $1^{+}23^{+}$, and the culture-induced suppressors of that response as Lyt $1^{+}23^{-}$.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: In addition to the effector cells generated in response to antigen stimulus, there has been increasing data presented to suggest that cell-mediated regulatory influences are also activated by antigenic stimulus. It has been the object of these studies to define the regulating influence governing the generation of T cell-mediated responses.

Methods Employed: T cell-dependent suppression of cell-mediated lympholysis was generated by in vitro culture of normal murine spleen cells.

I. The cellular requirements for generation of T cell-dependent suppression were studied by: 1) examining the effect of pretreating normal spleen cells with anti-Ia reagents and C on their ability to generate suppressors, 2) fractionating spleen cells by passage over Sephadex G-10 columns and analyzing the suppressor activity in column-passed populations; and 3) employing treatment with cytotoxic monoclonal anti-Lyt reagents to characterize the T cell subpopulation involved.

II. The ability of different inbred mouse strains to generate suppressor activity in vitro was compared.

Major Findings: I. Activation requirements for culture-induced generation of suppressor cells. It has previously been established that the generation of culture-induced T suppressors requires the participation of Ia positive radiation resistant splenic adherent cells. In order to study the mechanisms of "culture-induced" suppressor activation, it was tested whether or not there is a requirement for heterologous protein in suppressor cell induction. Culture in the presence of normal mouse serum was as effective in generating T suppressor cells as was culture in the presence of fetal calf serum. These findings are consistent with the interpretation that suppressor cell activation occurs spontaneously after removal from the regulatory influences of the in vivo environment.

In order to determine the identity of the T cell subpopulation(s) participating in the generation of these T suppressor cells, the effects of treatment with cytotoxic anti-Lyt hybridoma reagents were studied. Treatment of spleen cells either prior to or following in vitro culture demonstrated that Lyt 1^+23^- T cells are required for the suppression of cytotoxic responses by Lyt 1^+23^+ T cells.

II. Strain differences in the ability to generate suppressors. BALB/c ($H\text{-}2^d$) and B10 ($H\text{-}2^d$) spleen cells generated significantly more active suppressor populations than B10-D2 ($H\text{-}2^d$) spleen cells. In mixing experiments, it was found that BALB/c suppressors were efficient in suppressing the cell-mediated cytotoxic response of B10.D2 cells, while B10.D2 suppressors were not effective at suppressing BALB/c responses. The defect thus appears to be in the ability of B10.D2 cells to suppress, rather than in a difference in susceptibility to suppression.

Significance to Biomedical Research and the Program of the Institute: States of in vivo tolerance or immune hyporesponsiveness have been associated with active suppressive populations in a number of systems. In addition, the possible role of specific or nonspecific immune suppression in the evaluation or progression of malignancy has been suggested. The studies described above may provide information concerning the mechanism of such in vivo immunosuppression and possible means of modifying the suppression as a therapeutic manipulation of the immune response.

Proposed Course of Project: Further studies are in progress to determine the function of Lyt $1^{+}23^{-}$ T cells as suppressor inducers or suppressor effects, and the mechanism by which they suppress the cytotoxic T cell response.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05058-06 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunoregulation of Antibody Synthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. B. Dickler	Senior Investigator	I NCI
Other:	H. Weissberger	Postdoctoral Fellow	I NCI
	R. Shenk	Clinical Associate	I NCI

COOPERATING UNITS (if any)
none

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	2.25	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies specific for the idiotype(s) of anti-(T,G)-A--L antibodies of two different mouse strains (C57Bl/10Sn and C3H.SW) have been obtained. The idiotype(s) of these two strains are distinct and the anti-(T,G)-A--L antibodies of the majority of individuals of each strain express identical or cross-reactive idiotype(s). Since the ability of mice to respond to (T,G)A--L is under the control of H-2 linked Ir genes, these anti-idiotype antibodies are being used in conjunction with immunoassay of the binding of (T,G)-A--L to anti-(T,G)-A--L antibodies as well as both in vitro and in vivo humoral response assays to evaluate the cellular and genetic control of idiotype expression as well as the role of idiotype-bearing molecules in regulation of the immune response. T lymphocytes express anti-(T,G)-A--L idiotype(s) identical to or cross-reactive with those found on anti-(T,G)A--L antibodies of the same strain. Expression of similar or identical idiotypes is found on both IgM and IgG antibodies, and expression of these idiotypes is controlled (at least in part) by genes linked to the heavy chain allotype locus.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: 1. Determine the genetic control of idiotype expression on anti-(T,G)-A--L antibodies.

2. Determine the immunocompetent cell types which express idiotype bearing molecules (receptors).

3. Evaluate the role of idiotype-bearing molecules and cells in regulation of the immune response.

Methods Employed: 1. Preparation of anti-idiotypic antisera by immunization of Lewis rats with antigen affinity purified murine anti-(T,G)-A--L antibodies and adsorbtion on normal mouse globulins.

2. Radioimmunoassay that measures the binding of radiolabelled (T,G)-A--L by anti-(T,G)-A--L antibodies. Anti-idiotypic activity is measured as inhibition of binding of antibody to (T,G)-A--L.

3. Enzyme-linked immunoabsorbant assay (Elisa) which measures the binding of (T,G)-A--L specific antibody in (T,G)-A--L coated microtiter plates. Anti-idiotypic activity is measured as in No. 2.

4. Immunization of mice of different strains, including congenic resistant and recombinant inbred lines, with (T,G)-A--L and determining via immunoassay which mice bear idiotypes defined by these antisera.

5. Several antigen specific immune response assays including: a) in vitro primary antibody forming cell assay, and b) in vitro secondary antibody synthesis assay.

Major Findings: 1. The following antisera have been produced: Lewis anti-[C3H.SW anti-(T,G)-A--L] and Lewis anti-[B10 anti-(T,G)-A--L]. After adsorbtion on normal globulin from C3H.SW and B10 mice, respectively, the antisera have been shown to be directed against idiotypic determinants by a variety of criteria. The idiotype(s) of these two strains are distinct, as measured in the soluble phase radioimmunoassay, and the anti-(T,G)-A--L antibody of the majority of individuals of each strain express identical or cross-reactive idiotype(s).

2. Genetic studies with allotype congenic strains revealed that the expression of anti-(T,G)-A--L idiotypes is controlled by genes linked to the heavy chain allotype locus. These studies do not exclude regulation by other genes. Similar or identical idiotypes are present on primary IgM antibodies and secondary IgG antibodies.

3. Using a previously described primary in vitro antibody forming cell, it has been found that the Lew anti-[B10 anti-(T,G)-A--L] sera inhibits the response to (T,G)-A--L-TNP but not KLH-TNP in B10 mice, but not in C3H.SW or A.BY mice. Mixture experiments in which T lymphocytes from another $H\text{-}2^b$

strain (A.BY) were mixed with B cells from B10 did not show inhibition of the response by the anti-B10 idiotype. Also, treatment of B10 T lymphocytes with anti-idiotype antibodies and complement abrogates the ability of such cells to cooperate with B lymphocytes and accessory cells in the response to (T,G)-A--L-TNP. These studies taken together strongly suggest that T helper lymphocytes specific for (T,G)-A--L possess idiotypes identical or cross-reactive with those present on anti-(T,G)-A--L antibodies.

Using the newly developed recombinant inbred strain B10.MBR ($H\text{-}2K^b$, I^k) the Ir gene for (T,G)-A--L has been mapped to the I-A subregion.

Using the Elisa assay we have been able to obtain antigen-specific, T lymphocyte dependent responses to (T,G)-A--L in vitro. This is the first known success at obtaining an antibody response in vitro to an Ir gene regulated antigen. The characteristics of the system are as follows: 1) In order to detect such responses it is necessary to wash the antigen out of the cultures after 3 days and use a very sensitive detection system (Elisa); 2) Primed T lymphocytes are required; 3) The response is antigen-dependent and specific; 4) The response is under Ir gene control and the Ir genes are phenotypically expressed by B lymphocytes and/or accessory cells; 5) T and B lymphocytes can cooperate to produce these responses if they are H-2 identical even if non-H-2 is different. Thus, this system is ideally suited to carry out the objectives of this project.

Unfortunately, the Elisa assay utilized reveals cross-reactive idiotypes between mouse strains which differ at the heavy chain allotype locus. Experiments are in progress to determine if these cross-reactive idiotypes can be absorbed and if sufficient strain specific idiotypes remain to carry out the objectives of the project.

Significance to Biomedical Research and the Program of the Institute: Since idiotypes define a single or small number of clones of antibody, the determination of the genetic control of idiotype expression bears directly on the origins of diversity of antibody. An understanding of the genetic and cellular control of expression of idiotype bearing molecules (including antibody and T lymphocyte receptors) will clarify the role of such molecules in regulation of the immune response. An understanding of the regulation of the immune response could lead to new forms of therapy for human diseases including cancer.

Proposed Course of Project: 1. Evaluate the role of genes other than allotype linked genes in determining expression of anti-(T,G)-A--L idiotype(s) on antibodies.

2. Evaluate T lymphocytes and accessory cells for expression of anti-(T,G)-A--L idiotype(s).

3. Map the genes which determine expression of anti-(T,G)-A--L idiotype(s) on T lymphocytes (? and accessory cells).

4. Evaluate the role of other cell types (e.g. thymic epithelium) in the expression of idiotypes by T lymphocytes.

5. Evaluate the role of T lymphocytes in regulation of idiotype expression on antibody.

Publications

Markman, M. and Dickler, H. B.: Definitive mapping of the immune response gene(s) for (T,G)-A--L to the I-A subregion. Immunogenetics. 10: 93-96, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05062-06 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Application of Rapid Flow Microfluorometry to Cell Biology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	S. O. Sharrow	Chemist	I NCI
OTHER:	B. J. Mathieson	Staff Fellow	LMI NIAID
	S. Broder	Senior Investigator	M NCI
	D. G. Poplack	Senior Investigator	PO NCI
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	J. Smolen		NIAMD
	W. E. Biddison	Expert	I NCI
	G. M. Shearer	Senior Investigator	I NCI
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continued next page

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	0.2	2.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using rapid flow microfluorometry for analysis and sorting of cells, aspects of the following major subject areas have been supported during the previous year: (1) expression of cell surface differentiation markers on subsets of human T lymphocytes; (2) analysis of murine T cell differentiation markers during fetal development, during thymus repopulation following irradiation, and following enzymatic stripping from cell surfaces; (3) analysis of IgM allotypes and their genetic control; and (4) analysis of antibodies induced by anti-idiotypes.

PHS-6040
(Rev. 2-81)

A. Singer	Senior Investigator	I NCI
J. R. Ortaldo	Senior Investigator	LID NCI
R. B. Herberman	Chief, LID	LID NCI
U. Hammerling	Sloan Kettering Inst.	
J. Bluestone	Staff Fellow	I NCI
D. Sachs	Acting Chief, IB	I NCI
J. Kung		NIAID
W. Paul		NIAID

Project Description

Objectives: The objective of this project is to utilize rapid flow microfluorometry (FMF) to study selected aspects of important problems which would be impossible or extremely difficult to pursue without such technical support.

Methods Employed: Cells have been both analyzed and separated by rapid flow microfluorometry (Fluorescent-Activated Cell Sorter (FACS), Becton-Dickinson Electronics Laboratory, Mountain View, CA). These studies involve preliminary treatment of suspensions of viable, dispersed cells with purified, well-characterized fluorescent reagents.

Major Findings: This report summarizes the major thrust of each overall project emphasizing those aspects most heavily supported by use of rapid flow microfluorometry (FMF).

A variety of independent projects have been supported during the last year which are charting the differentiation antigens expressed by groups of human peripheral blood cells with differing functions. The subsets of T lymphocytes have been of particular interest: helper cells, suppressor cells, cytotoxic cells and their precursors, autorosette forming cells, and a close relative of T lymphocytes--natural killer cells.

Dr. Broder and colleagues. A change in cell surface differentiation determinants following an induced change of inactive precursors to suppressor T cells has been documented by FMF. This study was facilitated by identification of human leukemia T cells which in the presence of a T cell inducing factor, differentiate into cells with suppressor cell activity. The induced leukemia cells suppress pokeweed mitogen induced immunoglobulin production by normal human B cells. Five different monoclonal antibodies which react with functional subsets of human T cells have been used in a two-step labeling procedure to identify differentiation antigens on the induced suppressor leukemia cells and their inactive precursors. The most obvious change associated with acquisition of suppressor cell activity is expression of the "Tac" determinant, which in other studies has been associated with activated helper, suppressor and cytotoxic human T cells generated from normal donor blood cells.

Ms. Titus, Dr. Segal, and colleagues. The distribution of Fc receptors (FcR) for IgG immunoglobulin among human peripheral blood monocytes and T lymphocytes has been determined using FMF. Quantitative analysis of FcR expression has been facilitated by using affinity cross-linked rabbit IgG dimers and a fluoresceinated anti-rabbit IgG. Among T cells, this probe identifies the T cell subset commonly associated with suppressor cell activity. FcR expression has been correlated with populations of T cells and monocytes separated from peripheral blood by a variety of procedures. T cells have been isolated by both spontaneous rosette formation and by staining with a rhodamine-labeled hybridoma antibody which reacts with about 80% of peripheral blood T cells but not monocytes. The latter procedure has been used with 2-color FMF which allows simulateous identification of T-cell markers + FcR on single cells. Monocytes have been isolated by adherence, iron phagocytosis and density gradient separation. Analysis of FcR expression by the isolated cell populations shows that monocytes express a relatively low number of FcR per cell (1.3×10^4, average). Some T cells express relatively high numbers of FcR per cell (4.5×10^4, average), whereas the other T cells lack detectable FcR for IgG.

Dr. Ortaldo and colleagues. Cell surface determinants of another T cell related class of human peripheral blood lymphocytes have been analysed, namely the large granular leukocytes (LGL), which are closely associated with natural killer cell activity. Peripheral blood cells have been separated into T cells, monocytes, polymorphonuclear cells and LGL by density gradients, adherence and rosette formation. Using a panel of 17 reagents including 16 monoclonal antibodies which identify cell surface differentiation determinants, we found that the enriched LGL cells express a pattern of determinants which includes some overlap with classic peripheral blood T cells, some overlap with monocytes, some overlap with polymorphonuclear cells, but one determinant which appears selective for the LGL cells relative to other peripheral blood leukocytes.

Dr. Biddison and colleagues. FMF has also facilitated characterization of differentiation antigens expressed by human T lymphocyte subsets which participate in cytotoxic cell (CTL) responses against human influenza virus. Three monoclonal antibody reagents have been used to demonstrate with FMF that CTL and their precursors express determinants different from those expressed by at least one set of T helper cells. Two subsets of T helper cells have been identified, one of which has not been previously described.

Drs. Smolen, Steinberg and colleagues. Eight different monoclonal antibodies have been used to type human T cells which form autorosettes and those which do not. To date, non-rosette forming cells cannot be distinguished by expression of differentiation antigens from autorosette forming T cells, of interest as a model for self-recognition.

Dr. Mathieson and colleagues. Previous FMF work identified a new type of thymocyte present during the maturation of fetal mouse thymocytes. Thus, thymocytes defined by their expression of an Lyt 1^+ $(23)^-$ pattern of cell surface differentiation antigens, appeared at 14-16 days of gestation, which was up to 3 days before the appearance of Lyt(123)+ lymphocytes, a cell type

classically associated with adult thymocytes. The question was posed whether two separate T cell lineages exist (with the appearance of Lyt(123)+ cells possibly due to an influx of cells from outside the thymus) or whether Lyt(123)$^+$ cells are derived from Lyt 1$^+$ (23)$^-$ cells. To partially address this issue, thymocytes from thymus grafts between Lyt 2 congenic mice were monitored with monoclonal antibody reagents during the period of fetal development when transition of the Lyt phenotype occurs. The transition of Lyt phenotype occurred among the grafted cells, showing that Lyt(123)$^+$ cells can arise from pre-existing thymocytes and not necessarily from an influx of cells from outside the thymus.

Ms. Sharrow and colleagues. Previous FMF work showed that the thymus is initially repopulated by radio-resistant host cells following irradiation. The question of where such cells arise has been addressed, again using thymus transplants between mice congenic for Lyt antigen expression (which allows identification of thymocytes as donor thymocyte or host cell in origin). Analysis of thymocytes appearing early after irradiation shows that they arise from a radio-resistant population within the thymus.

Dr. Hooge and colleagues. FMF has also been used to study the turnover of Lyt antigens on mouse thymocytes following treatment of cells with trypsin. Lyt 2 and Lyt 3 antigens, like the Ly 9 antigen which recognizes all lymphocytes, have been easily removed with the exception of the Lyt 3.1 allele, which is partially resistant to trypsin. These characteristics were independent of donor strain. Cells were also cultured following trypsin treatment and analyzed for re-expression of cell surface antigens. Lyt antigens are expressed at normal levels after 24 hrs in culture; however, Ly 9 antigens are expressed at only half normal levels by that time. Analysis of time course data reveals symetrical unimodal peaks in the fluorescent distribution patterns, showing that major subpopulations of cells do not re-express antigens much faster than other subpopulations. Analysis of the turnover of thymocyte differentiation markers (Lyt) following trypsin treatment confirms that these markers are generated by thymocytes and not simply acquired from the circulation; it also confirms the existance of an Lyt 1$^+$(23) subset of thymocytes and exemplifies the use of FMF for quantitative turnover studies.

Drs. Kung, Paul, and colleagues. FMF has been used to characterize rat monoclonal antimouse IgM antibodies, one of which identifies a new allotypic determinant on mouse IgM. Three different levels of IgM allotype expression have been identified, which are under genetic control linked to the immunoglobulin heavy chain constant region complex.

Drs. Bluestone, Sachs, and colleagues. FMF has been used to quantitatively assess levels of antimouse MHC(H-2K^k) antibodies which are induced by injecting mice with antibodies (anti idiotypes) against monoclonal anti-mouse H-2K^k. Results show that 1) the anti H-2K^k antibodies induced by anti idiotype express idiotypes closely related to those of the original monoclonal anti H-2K^k antibodies against which the anti idiotype was raised, 2) induced anti H-2K^k idiotypes were associated with more than one subclass of immunoglobulin, 3) some induced immunoglobulin expressed the idiotype but not anti H-2K^k activity, and 4) anti idiotypes induced by different anti H-2K^k monoclonal antibodies can recognize unique determinants.

Significance to Biomedical Research and the Program of the Institute: Characterization of differentiation antigens expressed by functionally distinct subsets of lymphocytes will permit accurate enumeration of these cells and correlation of changes with disease states, will facilitate purification of these cells, and will further the investigation of the roles of the subsets in immune responses against tumors, viruses, organ grafts and auto-antigens. T cell differentiation markers have also been used to identify pathways for the development of murine T lymphocytes, which is an important step in understanding the role of these cells in immune responses and how that role might be manipulated.

Proposed Course of Project: As in the past, rapid flow microfluorometry will be used for selected, appropriate projects.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05064-05 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Genetic Control of the Immune Response In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Singer	Senior Investigator	I NCI
Other:	R. J. Hodes	Chief, Immunotherapy Section	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Requirement for self-recognition by T-helper cells of macrophage and B cell Ia determinants has been analyzed in vitro and in vivo. The self-recognition repertoires of T cells from A-->AxB, AxB-->A, and A-->B radiation bone marrow chimeras were restricted to the recognition of host, not donor, MHC determinants. While activation of T-helper cells invariably required self-recognition of macrophage Ia determinants, T-helper cell activation of B cells did not necessarily require T cell recognition of B cell Ia determinants. Indeed, it was found that those B cell responses which require T cell recognition of B cell Ia determinants were mediated by the Lyb5-B cell subpopulation, whereas B cell responses which were not T-B restricted involved the Lyb5+ B cell subpopulation.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The major objective of this project is the elucidation of the signals which are involved in the cell interactions which result in activation and/or regulation of the antigen-specific immune response.

Methods Employed: Chimeric mice are created by reconstituting lethally irradiated recipients with T cell depleted bone marrow stem cells.

The in vitro assay for antibody production involves dispersing single cell suspensions of murine spleens in microculture with TNP modified protein antigens for 4 days. The cells are then harvested and assayed for anti-TNP antibody-producing cells.

Spleen cell subpopulations are prepared as follows:

a) T cells-nylon non-adherent spleen cells

b) (B + accessory) cells - rabbit anti-mouse brain + C' treated spleen cells

c) Accessory cells - 2 hour glass adherent, T cell depleted, irradiated spleen cells

d) B cells - G-10 Sephadex passed, T cell depleted spleen cells

e) (T+B) cells - G-10 Sephadex passed spleen cells.

Major Findings: For responses to TNP-KLH, T-helper cells from A-->AXB, AxB-->A, and A-->B radiation bone marrow chimeras are only able to cooperate with macrophages expressing MHC determinants syngeneic to those of the chimeric host. These experiments demonstrate that activation of T-helper cells requires recognition of macrophage MHC determinants and that the specificities T cells recognize are determined by the environment in which they differentiate. In order to assess the possibility that T-helper cells recognized the same I region gene products as detected by antibodies, the ability of anti-I-A specific reagents to interfere with T-cell activation was assessed. Indeed, anti-I-A reagents specific for macrophage I-A determinants did block T-helper cell activation. Furthermore, experiments with restricted F_1-->parent chimeric T cells demonstrated that this was due to specific interference with the ability of T cells to recognize macrophage I-A determinants.

In addition, it was also found that T-helper cells could activate both Lyb5+ and Lyb5- B cell subpopulations, but that the activation of these two subpopulations was genetically distinct. Specifically, it was found that T cell activation of Lyb5- B cells was genetically restricted while T cell activation of Lyb5+ B cells was not genetically restricted.

Significance to Biomedical Research and the Program of the Institute: The regulation of the immune response by manipulation of the determinants actively recognized by cells as "self" promises to not only signficantly expand our understanding of the generation of the T cell repertoire and the requirements for B cell activation, but also promises to have a significant impact on our ability to regulate recognition of foreign antigen for the treatment of human disease.

Proposed Course of the Project: In the immediate future, the project will be directed at determining how self recognition by T and B cells is determined.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05065-05 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunologic Function of Accessory Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. B. Dickler Senior Investigator I NCI

COOPERATING UNITS (if any)

none

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long term goal is to explore the mechanism by which accessory cells process and present antigen to lymphoid cells and how they function in the regulation of the immune response. Using antigen-specific primed lymph node T cell proliferation and the in vitro primary antibody-forming cell response, the population(s) of cells possessing accessory cell function have been characterized: Thy 1.2 negative, surface Ig negative, I-A and I-E/C subregion antigens positive, radioresistant, glass adherent, phagocytic, Fc receptor positive. Accessory cell function required living cells and the efficiency of such function was directly related to the length of time the cells were exposed to antigen. Response to the antigens TNP-(T,G)-A--L and TNP-(H,G)-A--L was controlled by autosomal dominant genes located in the K or I-A regions of the responder H-2 complex and therefore mapped identically to the Ir genes controlling overall in vitro and in vivo responsiveness to these antigens.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To phenotypically characterize the cell population(s) which possess accessory cell function. 2) To evaluate the role of Ia antigens on accessory cells in terms of the activation of subsets of B and T lymphocytes. 3) To determine the relationship between accessory cell Ia antigens and the expression of Ir genes in these cells. 4) To explore the mechanisms by which accessory cells take up, "process" and present antigen to lymphoid cells.

Methods Employed: 1) Adherent cells are purified from spleen and peritoneal exudate by adherence to glass and removal or inactivation of other cell types by various means including: a) treatment with anti-Thy 1.2 and C', b) irradiation, c) fluorescein-conjugated $F(ab')_2$ anti-Ig or fluorescein-conjugated protein coated polystyrene particles plus fluorescence activated cell sorting. 2) Accessory cells are removed from lymphoid cell populations by passage over Sephadex G10. 3) Ia antigens and other surface markers are detected by immunofluorescence. Ia bearing cells are removed by treatment with alloantisera and C'. The synthesis of Ia antigens is measured by internal labeling with ^3H-leucine, followed by solubilization, immunoprecipitation and gel electrophoresis. 4) Fc receptors were evaluated using antigen antibody complexes and indirect immunofluorescence. 5) The immunologic function of accessory cells is assessed by T cell activation as measured by thymidine incorporation in vitro, or by antibody formation by B cells in the in vitro induction of primary antibody-forming cell responses as measured by plaque formation.

Major Findings: T lymphocyte dependent antigen specific immune responses were evaluated using lymphocyte populations which required the addition of exogenous accessory cells in order to phenotypically characterize the cell(s) which provide accessory cell function. This function was provided by a population which was Thy 1.2 negative, radioresistant, glass-adherent, and were only functional if alive. The accessory cell function of spleen adherent cells was proportional to the length of time such cells were incubated with antigen and very small numbers of such cells provided accessory cell function. Depletion of surface Ig positive cells from the adherent population did not affect accessory cell function whereas depletion and reconstitution experiments indicated that phagocytic cells were required. Such cells also bore Fc receptors. Cytotoxic studies with subregion restricted anti-Ia antibodies and complement indicated that accessory cell function resided in a subpopulation of spleen adherent cells which bore the I-A and I-E or C subregion antigens. The function of such cells was not related to a selective ability (vs. other spleen adherent cells) to take up antigen.

The possibility was investigated that Ir genes regulate the function of cells other than T or B cells in the primary IgM PFC responses to the synthetic antigens TNP-(T,G)-A--L and TNP-(H,G)-A--L. The primary PFC responses of (Responder x Nonresponder)F_1 spleen cells to both antigens were abrogated by G-10 Sephadex passage and restored by the addition of spleen adherent

cells which had the characteristics described above. The abrogated responses of G-10 Sephadex passed (Responder x Nonresponder)F_1 spleen cells to each antigen were reconstituted only by spleen adherent cells from stains which were responders to that antigen. All the spleen adherent cells tested restored the non-Ir gene controlled response to a third antigen, TNP-KLH. The ability of spleen adherent cells to function as accessory cells in the primary PFC responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L was shown to be controlled by autosomal dominant genes which were shown by the use of recombinant strains to be located in the K or I-A regions of the responder H-2 complex, the same region(s) of H-2 as the Ir genes controlling overall in vitro and in vivo responsiveness to these antigens.

Significance to Biomedical Research and the Program of the Institute: Since spleen adherent cells function as accessory cells in antigen presentation to T lymphocytes and phenotypically express both Ia antigens and Ir genes, an understanding of the mechanisms underlying accessory cell function in the immune response may lead to new modes of therapy for cancer and other human diseases.

Proposed Course of Project: Due to time commitments to other projects this study will not be pursued at this time. However, when time and personnel permit, the proposed course will be: 1) Evaluation of the functional interactions between subpopulations of adherent cells and subpopulations of lymphocytes; 2) The mechanism of Ag uptake and processing by accessory cells will be examined by internal and surface labeling of Ia in conjunction with uptake of radiolabelled antigens under Ir gene control.

Publications

Dickler, H. B., Cowing, C., Ahmann, G. B., Hathcock, K. S., Sachs, D. H., Hodes, R. J., and Singer, A.: Expression of Ir genes and Ia antigens by adherent accessory cells required for antigen-specific antibody forming cell responses. In van Furth, R. (Ed.): Mononuclear Phagocytes Functional Aspects. The Hague, Martinus Nijhoff, 1980, pp 1909-1922.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05067-06 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic Control of Human in Vitro Cellular Immune Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Shaw	Investigator	I	NCI
	W. E. Biddison	Expert	I	NCI
Other:	G. M. Shearer	Senior Investigator	I	NCI
	D. Nelson		MET	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies are continuing on the role of HLA gene products in recognition of foreign antigen by human T cells. Studies in this project with the hapten TNP complements the studies of influenza virus in project Z01-CB-05078 I. The principal effector mechanism under investigation is cell-mediated cytotoxicity, but proliferation is also used as a measurement of T cell response. Conditions have been optimized to allow use of frozen peripheral blood lymphocytes as responders, which will facilitate further immunogenetic analysis. More careful analysis of the TNP-restriction antigens by direct lysis and blocking confirms involvement of B cell antigens, probably including, but not restricted to HLA-DR. Monoclonal antibody inhibition studies are in progress as another tool in defining the restriction antigens involved.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The primary objectives of this laboratory are to investigate the function of T lymphocytes, the role of MHC antigens in self recognition, and the mechanism by which these genes control immune responses. The hypotheses generated draw heavily from the precedents in animal models, particularly those in the mouse, but the experimental work is restricted to human studies. Because of the ethical and logistical considerations which limit in vivo studies in humans, it has been (and is) crucial to develop good in vitro models of human immune responses. Two model systems have been chosen for current investigations: cytotoxic T cell responses to TNP (included in this project), and influenza virus (project Z01-CB-05078 I). Both systems have their advantages. The advantages of the TNP system are that there is more flexibility in introducing the antigen on the cell surface, and that since it is not a usual environmental antigen it is possible to analyze responses in unsensitized donors.

Methods Employed: Human PBL were obtained from donors by phlebotomy or batch leukapheresis; mononuclear cells are prepared by density separation, and cryopreserved. Stimulator cells are covalently modified with TNP, and cytotoxic effector function studied after two cycles of in vitro stimulation. Cytotoxic activity is analyzed by short term ^{51}Cr release assays using as targets T lymphoblasts or lymphoblastoid B cell lines which have been TNP modified. Analysis of the HLA markers on the donor cells is performed by microcytotoxicity testing under contract N01-CB-04337.

Major Findings: Previous studies in the TNP system were performed using fresh responder and stimulator cells, in a culture media containing autologous plasma; these conditions were adopted to optimize the strength of the response and to minimize possible artifacts introduced by allogeneic plasma proteins. We have subsequently optimized the system using exclusively frozen cells and pooled human plasma. The introduction of allogeneic proteins has had no demonstrable effect on the specificity of this "modified self" response. Using the incresed flexibility made possible by these improvements, we have continued studies of recognition of B cell antigens in association with TNP. Direct lysis and cold target inhibition studies confirm that some cytotoxic T cells recognize TNP in conjunction with self antigens preferentially expressed on B cells. HLA-DR antigens per se appear to contribute some to this recognition, but HLA-DR (as currently defined serologically) cannot explain all of the recognition observed. Initial studies have been performed using monoclonal antibody inhibition of CML as a probe to analyse the self antigens which are recognized in conjunction with foreign antigen. Studies are incomplete, but suggest that the unusual recognition of TNP in association with antigens widely shared between humans may not be inhibitable by a monoclonal antibody directed at framework structures on the HLA-A,B,C molecules.

Significance to Biomedical Research and the Program of the Institute: The problems addressed in this project are central to the understanding of how the immune system surveys the body to detect foreign antigen and to eliminate cells which express those foreign antigens. This surveillance system is thought to be important not only in dealing with microbial pathogens but also in detection and destruction of neoplastic cells. Furthermore, these studies promise to clarify the relevance of genetic differences between individuals in their susceptibility to infectious, neoplastic and autoimmune diseases.

Proposed Course of the Project: The studies of hapten-modified cells (this project) and virus-infected cells (project Z01-CB-05078 I) will be continued in parallel because of the informative similarities and differences in the two systems. The primary thrust in the TNP system will be twofold: 1) To clarify the nature of the unusual CML restriction antigens in this system. T cell recognition of HLA-linked B cell antigens and of relatively nonpolymorphic antigens in conjunction with TNP is unique to this in vitro system, and may provide the exception which "proves" the rule regarding which self antigens can function in associative recognition of foreign antigen. Studies of the polymorphic cell surface antigens can be performed using carefully chosen combinations of effector and target cell donors. In addition, all classes of restriction antigens can be studied by monoclonal antibody inhibition studies. 2) To study the nature of the interaction between antigen and cell surface which is necessary to trigger an immune response; this is facilitated in the TNP system because of the flexibility inherent in chemical modification of the cell surface. Studies may be carried out which are similar to those previously done in murine studies using specifically synthesized molecular probes to carry TNP onto the cell surface, and to study the immune response to these directed TNP moieties.

Publications

Biddison, W. E., Payne, S. M., Shearer, G. M. and Shaw, S.: Human cytotoxic T cell responses to trinitrophenyl hapten and influenza virus: diversity of restriction antigens and specificity of HLA-linked genetic regulation. J. Exp. Med. 152: 204s-217s, 1980.

Shaw, S.: Human T-cell-mediated cytotoxicity: The role of HLA. In Sell, K. (Ed.): The Lymphocyte. New York, Alan Liss, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05068-05 I

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunology of Model Membrane Macromolecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. A. Henkart Senior Investigator I NCI

COOPERATING UNITS (if any)
D. Wolf and W. Webb, Department of Applied Physics, Cornell University

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES x ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Synthetic amphiphilic macromolecules composed of stearic acid covalently linked to fluorescent dextran derivatives were synthesized as models for membrane proteins. These water-soluble molecules adsorb to all artificial and cell membranes tested. The lateral diffusion and qualitative behavior of these molecules on the surface of fibroblasts have been studied and artificial membranes. The lateral diffusion constant of these molecules on the surface of fibroblasts is about 2×10^{-10} cm^2/sec, and on synthetic membranes is about 5-fold faster. Addition of antibody to determinants on the dextran produces patching for all membrane bound molecules; for lymphocytes and for many fibroblasts, capping also occurs. In addition the technology developed was used to allow the formation of IgA coated erythrocytes which have successfully detected IgA specific Fc receptors on mouse lymphocytes.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To test the properties of synthetic membrane antigens in cell membranes and compare these with natural membrane antigens in the same cells. A molecular explanation of the capping phenomenon which occurs upon cross-linking membrane antigens is sought. Attempts to use defined soluble antigens as targets for T lymphocyte killing are being carried out.

Methods Employed: Dextran and other polysaccharide derivatives containing covalently linked TNP, rhodamine and sterate were synthesized. Saline solutions of these compounds (10-1000 g/ml) were incubated with cells followed by washing. The adsorbed antigen was then followed by using the fluorescence microscope with direct visualized or by staining with rhodamine labeled anti-TNP. Quantitative measurements of lateral diffusion were made using fluorescence photobleaching recovery. IgA coated ox erythrocytes were made by adsorbing stearoylated dextrans and arabinogalactans to the erythrocytes and then incubating with QUPC 52 and SAPC 10, IgA myeloma proteins which bind the respective polysaccharide. These red cells were incubated with lymphocytes and found to form rosettes by standard techniques.

Major Findings: Stearoylated dextrans adsorbed on the membrane of fibroblasts undergo patching and capping when crosslinked by antibody. These processes are remarkably similar to those seen with natural fibroblast antigens. On flattened cells, patches form in rows, very similar to those shown to correlate with intracellular myosin by other groups. A quantitative study of lateral diffusion of these molecules showed that they move at the same rate as the less restricted membrane proteins previously studied, i.e., with a lateral diffusion constant of 2×10^{-10} cm^2/sec. When crosslinked by low concentrations of antibody, the lateral diffusion is greatly retarded, even under conditions where no patches are visible. A continuation of the study of these molecules on lymphocyte surfaces showed that thymocytes absorbed these molecules from solution more readily than splenocytes, showed an optimum surface concentration for capping, and capped the adsorbed species more slowly than splenocytes. The drug inhibition of the capping on these cells, like stearoylated dextrans on spleen cells, was very similar to drug inhibition of capping of natural markers such as sIg. In the IgA Fc receptor collaborative study with Warren Strober in the Metabolism Branch, NCI, 4-6% of normal mouse spleen cells were found to form IgA rosettes, implying that this class of antibody has its own Fc receptor.

Significifcance to Biomedical Research and the Program of the Institute: This project is aimed at elucidating basic aspects of biological membranes, especially the behavior of antigens on cell surfaces. Such antigens play a vital role in graft and tumor rejection. The studies undertaken have revealed that simple model membrane molecules passively inserted into membranes show many of the properties of natural membrane antigens, including capping.

Proposed Course of Project: Both TNP and Fluorescein substituted stearage dextrans will be used to attempt to detect T lymphocyte recognition of cells bearing these synthetic antigens.

Publications

Wolf, D. E., Henkart, P., and Webb, W. W.: Diffusion, patching and capping of stearoylated dextrans on 3T3 cell plasma membranes. Biochemistry, 19: 3893-3904, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 05069-05 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Expression of Ia Antigens on Functional Cell Subpopulations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Nadler	Investigator	I NCI
Other:	R. J. Hodes	Chief, Immunotherapy Section	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	1.25	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been demonstrated that the T cell proliferative response to Con A and the T cell dependent IgM response to the soluble antigens TNP-KLH, TNP-T,G-(A-L), and TNP-Nuclease require the participation of accessory cells. This subpopulation of spleen cells has been shown to be glass adherent, radioresistant, non-T, non-B and and to express Ia (I region associated) determinants encoded in I-A and I-E/C. In addition, cells within the splenic adherent cell population are the predominant stimulators of the one way murine mixed lymphocyte response when responder and stimulator cells differ either at H-2 or the Mls locus. These stimulator cells also bear I-A and I-E/C encoded determinants and their ability to stimulate allogeneic T cells could be inhibited with specific anti-Ia reagents. Similar results regarding Ia antigen expression and functional accessory cell competance were also obtained using a population of purified murine Kupffer cells, the tissue macrophage of the liver.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Ia antigens are serologically demonstratable cell surface determinants which appear to play important roles in cell-cell interactions. The objective of this study is to investigate the role these Ia determinants play in cellular interactions involved in T cell-mediated or T cell-dependent responses. The T cell proliferative response to Con A and the T cell-dependent in vitro primary antibody response to TNP-conjugated proteins are being studied as well as the ability of various subpopulations of spleen to stimulate in an allogeneic mixed lymphocyte response.

Methods Employed: Cell separation and reconstitution techniques are being employed and have established that accessory cells are required for both the Con A proliferative response and the primary antibody response to TNP-KLH. Kupffer cells, the tissue macrophage of the liver were prepared by sequential enzymatic digestion of liver, differential sedimentation, glass adherence and treatment with RαMB + C. SAC and Kupffer cells were shown to be potent stimulators of the allogeneic one way mixed lymphocyte response in addition to serving as accessory cells for mitogen and antibody responses. Accessory populations were treated with anti-Ia reagents and complement (C) and assessed for their ability to reconstitute both responses. Accessory cells are also being fractionated into phagocytic and non-phagocytic populations by their ability to ingest fluorescent latex particles as detected by the Fluorescent Activated Cell Sorter and these fractions then evaluated for their ability to restore the Con A response and the in vitro primary antibody response.

Major Findings: (1) It has been shown that the T cell proliferative response to Con A and the T cell dependent primary in vitro antibody response to TNP-KLH require the presence of non-T, radio-resistant glass adherent spleen cells. In addition, cells within this subpopulation of cells (SAC or Kupffer cells) were shown to be potent stimulators of the murine mixed lymphocyte response. (2) Kupffer cells, the tissue macrophage of the liver were shown to be similar to splenic adherent cells (SAC) morphologically, in their expression of cell surface determinants (Ia antigens, Fc receptors and complement receptors) and functionally in the assay systems noted above. (3) Accessory cell function of these Kupffer cells and splenic adherent cells (SAC) is abrogated by treatment with anti-Ia and C. Using subregion specific anti-Ia reagents, it was shown that these cells expressed Ia determinants encoded by genes in I-A and I-E/C. In addition, these determinants are expressed on the same cells. (4) The reconstituting SAC in the Con A response were effective whether or not they were H-2 identical with the responding T cells. (5) Cells within both the SAC and Kupffer cell population were shown to be potent stimulators of the mixed lymphocyte response (MLR). The cells stimulating the MLR also bore determinants encoded in both I-A and I-E/C. Cell separation techniques showed that splenic B and T cells were poor stimulators of MLR when stimulators and responders differ at either H-2 or Mls. Depletion of splenic accessory cells by passage over G-10 Sephadex resulted in diminution of stimulatory ability in MLR whereas enrichment for accessory cells by adherence, irradiation and RαMB + C treatment resulted in a population significantly more stimulatory than unfractionated spleen.

Significance to Biomedical Research and the Program of the Institute: I region gene products play a significant role in regulation of immune responses. A basic understanding of the expression, and perhaps the functional role of one class of I region gene products, Ia antigens, in mitogen responses, and in antibody production should provide insight into the mechanisms of control of these immune responses. Such insight would be relevant to evaluation of the host response to allograft and tumor challenge.

Proposed Course of Project: Studies are in progress to further define the properties of the Ia^+ accessory cells and their mechanism of action in antibody responses under Ir gene control. Studies are planned to further evaluate the functional and/or structural significance of the finding that B cells which bear Ia determinants are not capable of triggering proliferation of allogeneic T cells whereas SAC bearing determinants which are serologically identical are potent stimulators of MLR.

Publications

Nadler, P. I., Klingenstein, R. J., Richman, L. K., and Ahmann, G. B.: The murine Kupffer cell. II. Function in in vitro primary antibody responses, mitogen induced proliferation and stimulation of a mixed lymphocyte response. J. Immunol. 125: 914-920, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05078 04 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to Influenza-Infected Autologous Lymphocytes in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. E. Biddison	Cancer Expert	I	NCI
	J. S. Shaw	Senior Investigator	MET	NCI
Other:	G. M. Shearer	Senior Investigator	I	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro stimulation of influenza virus-immune cytotoxic effector cells in human peripheral blood leukocyte (PBL) populations; and (b) measurement of effector cell activity on virus-infected cryopreserved human PBL target cells was investigated. Effector cells generated in vitro are influenza type-specific and are HLA-A and HLA-B restricted. The cytotoxic response appears to be controlled by HLA-linked immune response (Ir) genes since: (a) cytotoxic activity is predominantly associated with antigens of only one haplotype; and (b) effector cells from certain donors recognize virus in association with some but not all self HLA-A and -B antigens. This Ir gene control is HLAlinked and antigen-specific, since preferential recognition among HLA-identical siblings is different for influenza A and B viruses. Comparative studies of HLA-A positive and of HLA-A3 positive donors for these CTL responses indicate that greater heterogeneity of these HLA antigens can be demonstrated by CTL than by current serology. The CTL response to influenza virus was found to depend on interacting populations of helper and CTL precursor T cells.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The primary objectives of this laboratory are to investigate the function of T lymphocytes, the role of self recognition, and the effects of major histocompatibility genes on the murine and human immune systems. These studies are being pursued using mouse and human leukocytes which are sensitized to autologous cells either modified with chemical agents (e.g. the trinitrophenyl group) or infected with viruses (e.g. influenza, measles). The objectives of this project are to: (a) define the components of the human major histocompatibility complex (HLA) which are recognized in association with viral antigens by cytotoxic T cells; (b) analyze the virus specificity of human influenza immune cytotoxic T cells; (c) determine if there is any "Ir-like" genetic control of human T cell response potential to influenza viruses; (d) characterize the human cell populations required to generate virus-immune cytotoxic T cells; and (e) to determine whether different sites or epitopes on the HLA molecule can be identified which serve as self recognition structures for recognition of virus.

Methods Employed: Human PBL were obtained from normal adult volunteers, separated on a Ficoll-Hypaque gradient, and sensitized in vitro to live influenza viruses. Cytotoxic activity was measured by ^{51}Cr release from cryopreserved peripheral blood cells which were thawed, stimulated with PHA, and infected with influenza virus. The OKT series of monoclonal antibodies was used in association with the FACS II and cell separation by monolayers to positively and negatively select subpopulations of human T cells required for CTL responses to influenza virus.

Major Findings: The human cytotoxic T cell response in vitro to type A influenza viruses is predominantly directed against cross-reactive determinants on cells infected with serologically distinct type A influenza viruses. Reciprocal exclusion of cytotoxicity was observed between effectors sensitized to type A and B influenza viruses. Human influenza virus-immune cytotoxic T cells recognize viral determinants predominantly (>90%) in conjunction with self antigens that are encoded by genes closely linked to HLA in family studies and that are highly associated with the HLA-A and -B loci in population studies. In a family study, siblings consistently generated cytotoxic activity against influenza predominantly in association with antigens coded by genes of only one of their HLA haplotypes. Such haplotype preferences were consistent among HLA-identical siblings, indicating that the specificity of the T cell response to influenza virus in association with HLA-A and -B antigens is controlled by genes linked to HLA. Virus-immune effectors from certain donors recognize virus in conjunction with some, but not all, of their self HLA-A and -B antigens. Among donors who who share a given HLA antigen (such as A2 or B7), there are differences in the ability of their virus-immune T cells to recognize the shared antigen. Virus-infected target cells from HLA-A2 or B7 "nonresponder" donors could be lysed by virus-immune T cells obtained from other donors who shared only the HLA-A2 or -B7 antigen with these target cells. These observations suggest that the absence of cytotoxic T cell responses by some donors to influenza virus in conjunction with particular self HLA-A and -B antigens is not due to control by the structural genes which code for these HLA antigens, but rather may result from control of regulatory genes which act

at the level of the responder and/or stimulator cell. An individual with a variant HLA-A2 molecule has been detected by influenza-specific and allogeneic cytotoxic responses. This variant is not detectable by serotyping, but has been verified by HLA peptide analysis. Similar observations have been made in which variant HLA-A3 donors have been identified by influenza-specific CTL. Cell fractionation studies using the OKT series of monoclonal reagents indicate that influenza-specific CTL precursors are $OKT3^+$, $OKT4^-$ and $OKT8^+$, whereas helper T cells are either $OKT4^+$ or $OKT4^-$.

Significance to Biomedical Research and the Program of the Institute: This project is of fundamental immunological importance, since: (a) it provides one of the first known examples of cell-mediated immunity which involves self-recognition by human lymphocytes, and (b) it involves a virus which is of significance in human disease. Furthermore, this system can be used to test a number of major histocompatibility-linked immune phenomena in man which are known to occur in mice (e.g., MHC-restriction, haplotype preference, Ir gene control, and cell-to-cell interactions).

Proposed Course of Project: Further studies will be performed using lymphocyte populations from unrelated HLA matched and unmatched individuals, and from members of families including those known to be recombinants within the HLA complex in order to better define the components of the HLA complex which are recognized in association with viral antigens. Studies will be performed to examine antigen specificity of HLA-linked genetic control of virus-immune T cell specificity. Preliminary results suggest that there are differences in the HLA haplotype preferences observed between T cells sensitized to A/HK and B/HK influenza viruses. In population studies, there are differences between A/HK and B/HK-immune T cells in recognition by T cells may help to determine whether different repertoires for foreign antigens exist for T cells which recognize different self determinants. We plan to determine if differences in HLA recognition by virus-immune T cells are due to differences in the responder and/or stimulator cell functions. In order to use responder and stimulator peripheral blood leukocytes from different individuals for generation of virus-immune cytotoxic T cells, it is first necessary to remove any responder cells capable of generating an alloimmune response against the stimulator cells. To remove this alloreactive potential, we are developing a negative selection technique based on specific adherence of T-cells to monolayers of cells expressing alloantigens. We plan to investigate whether influenza virus can be recognized in association with different HLA-coded self determinants by attempting to block the cytotoxic reaction with monoclonal antibodies directed against distinct parts of HLA molecules. Studies will be continued using the OKT series of antibodies to identify other populations of T cells. Biochemical studies of the HLA-A3 variant donors will be made to determine whether there is molecular evidence for these variants.

Publications

Biddison, W. E., Krangel, M. S., Strominger, J. L., Ward, F. E., Shearer, G. M. and Shaw, S.: Virus-immune cytotoxic T cells recognize structural differences between serologically indistinguishable HLA-A2 molecules. Human Immunol. 3: 225-232, 1980.

Biddison, W. E., Payne, S. M., Shearer, G. M., and Shaw, S.: Human cytotoxic T cell responses to trinitrophenyl hapten and influenza virus: Diversity of restriction antigens and specificity of HLA-linked genetic regulation. J. Exp. Med. 3: 225-232, 1980.

Biddison, W. E., Ward, F. E., Shearer, G. M. and Shaw, S.: The self determinants recognized by human virus-immune T cells can be distinguished from the serologically defined HLA antigens. J. Immunol. 124: 548-552, 1980.

Biddison, W. E., Sharrow, S. O., and Shearer, G. M.: T cell subpopulations required for the human cytotoxic T lymphocyte response to influenza virus: evidence for help. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05081-04 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Ontogeny of Immune Responsiveness in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Nadler	Investigator	I NCI
Other:	R. J. Hodes	Chief, Immunotherapy Section	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ability of mammalian species to respond to in vivo challenges with complex antigens develops in a time dependent manner during embryonic and neonatal life. Functional or structural immaturity of B cells, T cells, and accessory cells in fetal or neonatal animals as well as active, non-specific suppression of humoral and cellular immune responses by neonatal immunocytes has been reported. Utilizing two systems, 1) the in vitro primary antibody response to soluble hapten-protein conjugates, and 2) the one way mixed lymphocyte response, it has been shown that thymic T cells and both splenic T and non-T cells are capable of actively suppressing immune response by cells from adult animals. The splenic adherent cell population (SAC) isolated from neonates was also shown to be an inefficient antigen presenting accessory cell. These neonatal SAC were also inefficient stimulators of MLR across both H-2 and Mls differences. Fluorescent microscopic studies revealed that fewer neonatal SAC were Ia positive than adult SAC. Moreover the development of functional accessory cell activity with age paralleled the expression of Ia antigens by SAC.

Project Description

Objectives: Investigation is under way concerning the maturation of the immune response in mice to soluble hapten-protein conjugates. Inability to respond to various antigens has been noted in many species during embryonic development and in the neonatal period. Spleen cells from 7 to 10 day old mice are unable to respond in vitro to soluble TNP-KLH as measured by a plaque forming cell assay of primary IgM antibody response. The mechanism of this neonatal unresponsiveness is the object of this project.

Methods Employed: The major experimental systems employed include in vitro primary antibody responses as measured by a plaque forming cell response, the one way mixed lymphocyte response and cell surface immunofluorescent labelling. Cell separation procedures include nylon non-adherence to obtain populations enriched in T cells; RαMB and complement treatment and G10 Sephadex passage to obtain populations enriched in B cells, G10 Sephadex passage to obtain macrophage or accessory cell depleted populations and glass adherence for macrophage or adherent cell populations.

Major Findings: The ability of spleen cells to respond to soluble TNP-KLH appears approximately 2-3 weeks after birth in BALB/c mice with inconsistent responses prior to this period. Responses appear maximal between 2-7 months of age. The nature of neonatal unresponsiveness in vivo and in vitro could result from active suppression in neonatal spleen cells and/or from defects in the functional capacity of one or more neonatal populations. In the present study, unfractionated neonatal spleen cells consistently suppressed antibody responses by adult spleen cells. Attempts to isolate a population containing the suppressor cell(s) led to the finding that both "T-enriched" (nylon-non-adherent) and "non-T" (RαMB+C treated and/or G10 passed, RαMB+C treated) populations could mediate this suppression. Treatment with T cell specific reagents (RαMB, αθ ascites and an αθ hybridoma) failed to abrogate the suppressor activity in neonatal spleens. Treatment of neonatal spleen with αIa antisera and C also had no effect on this active suppression. Studies aimed at determining the immune competence of subpopulations of neonatal spleen (e.g. T cells, B cells, and macrophages) were complicated by the presence of active suppressor cells in both the T and non-T cell subpopulation. The function of accessory cells could be investigated because of the relative radioresistance of accessory cell function and the relative radiosensitivity of the suppressor cell activity of neonatal spleen cells. The ability of neonatal and adult adherent spleen cells to function as accessory/antigen presenting cells was assessed for in vitro primary antibody responses to TNP-KLH. Neonatal splenic adherent cells (SAC) were shown to be less efficient antigen presenting accessory cells than adult SAC and this ability was shown to mature in a time dependent manner. It was demonstrated that fewer latex-phagocytic cells in the neonatal adherent spleen cell populations expressed cell surface Ia antigens than adult splenic latex-phagocytic cells. The expression of Ia antigens on latex-phagocytic cells progresses from the neonatal period into adult life and reaches plateau values approximately 6 weeks after birth. The appearance of an increasing proportion of Ia positive, latex-phagocytic cells in the SAC subpopulation was shown to

correlate highly with the ability of this population to function as antigen presenting accessory cells for in vitro responses to TNP-KLH. The observation that cells within the SAC population are the major stimulators of the murine mixed lymphocyte response (see Project # Z01-CB-05069-03 I) led us to investigate the ontogeny of cellular stimulatory ability in the MLR. As was observed for accessory and antigen presenting function, neonatal SAC were less potent stimulators of T cell proliferation than adult SAC when the stimulator and responder strains differed either at the Mls locus, the entire murine major histocompatibility complex (H-2) or subregions within H-2. The development of this ability to stimulate mixed lymphocyte responses across I region-encoded determinant differences paralleled the ontogenetic rate of expression of these antigens on SAC as examined by immunofluorescent techniques. The ability of neonatal and adult SAC to stimulate either allogeneic CML or "TNP-modified self" CML was comparable and fluorescent investigation of expression on K region encoded determinants on these cells revealed that the majority of both newborn and adult SAC bore these determinants. It was also confirmed that sIgM positive splenic (B) cells are virtually all Ia antigen positive by the end of the first postnatal week.

Significance to Biomedical Research and the Program of the Institute: It is known that immune responsiveness in neonatal and senescent animals is deficient relative to adult animals of that species. Information concerning the mechanisms of neonatal immune deficiency is incomplete and investigations in this area will be applicable to the process of differentiation and to possible means of modulation of responsiveness. It is also known that the incidence of malignant neoplasms is greatest at the extremes of life and it has been postulated that defects in "immunologic surveillance" may play a role in this occurrence. Studies of neonatal cellular competence and the mechanism of action of neonatal suppressor cells may shed light on these postulated defects. This work demonstrates that neonatal immunoincompetence is the result of a complex interplay of suppressor cell influence and immaturity of one or more of the cell population required for responsiveness. Evidence has been accumulated that the ontogenetic rate of expression of Ia antigens differs on different populations of cells involved in immune responses and that the expression of Ia antigen on cells within the SAC population is fundamental for their cooperation in antibody responses.

Proposed Course of Project: Continuing studies will pursue the cellular causes of the inability of neonatal animals to respond to soluble antigens. Specifically, the ability of neonatal SAC to provide accessory cell function for responses to T independent, macrophage dependent antigens like TNP-Ficoll will be investigated. In addition, means of inducing expression of Ia antigens on cells within the SAC population will be investigated.

Publications

Nadler, P. I., Klingenstein, R. J., and Hodes, R. J.: Ontogeny of neonatal accessory cells. Ia antigen expression and function in in vitro primary antibody responses. J. Immunol. 125: 914-920, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05083-03 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Genome Organization of Murine Major Histocompatibility Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. Singer	Senior Staff Fellow	I NCI
Other:	D. H. Sachs	Chief, Transplantation Biology Section	I NCI
	S. Rudikoff	Senior Investigator	LCB NCI
	L. Abelson	Biologist	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	5.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES x ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study was initiated to determine the DNA sequence organization of the genes encoding the major histocompatibility complex and the mechanisms controlling the expression of these genes.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: This laboratory has undertaken to study the molecular biology of the major histocompatibility locus in the mouse and the miniature swine. In both species, it is known that the structural products regulated by this region of the genome are highly polymorphic and responsible for both transplantation rejection and regulation of immune responses. Biochemical studies have demonstrated that the MHC antigens of mouse and miniature swine are structurally homologous.

Genetic studies have shown that the genes involved in regulating the expression of the transplantation antigens, as well as those involved in regulating the immune response are all linked on a single chromosome. However, the organization of these genes varies between the two species. Therefore, this region is of considerable interest in studying the regulation of a coordinated set of functions. The objects of the studies are to characterize the genome organizations and regulation of these families of genes in each of the two species and to analyze the evolutionary relationship between them.

Methods Employed: Two separate approaches are being taken to attempt to purify the genes of these MHC loci. In the first, the mRNA species encoding MHC products are being purified from the spleens of each of the species under study. The purification is monitored in two ways: by the ability of various mRNA fractions to direct the cell free synthesis of the structural products and by their ability to hybridize to a heterologous human MHC probe. The purified mRNA species will then serve as templates in the synthesis of double stranded cDNA which will be propagated in recombinant bacterial plasmids.

The alternative approach to studying the genomic organization of the MHC locus involves the direct isolation of the individual genes encoding the various structural products. Total mouse DNA, isolated from the livers of inbred strains of mice and miniature swine, is enzymatically fragmented, and each fragment is inserted into a viral vector. These recombinant DNA libraries are then screened using a heterologous human MHC cDNA probe. Isolated genomic clones are characterized either by direct DNA sequence analysis or by the ability of these genes to direct the synthesis of MHC products. The isolation of such genomic MHC genes will allow an analysis of the organization of a single constitutively expressed gene, as well as its relationship to other members of a multigene family.

Major Findings: Through analysis of splenic RNA by molecular hybridization, it has been determined that the RNA species encoding transplantation antigens are all approximately 16S in size. In contrast, in the pig there appear to be multiple RNA species homologous to MHC genes. The major species is approximately 14S, somewhat smaller than the murine MHC RNA. The remaining species are all larger. The relationships between these different molecules remains to be determined.

Studies on the in vitro synthesis of MHC products have yielded new insights into the biogenesis of MHC products. PolyA$^+$-RNA was isolated from mouse spleen, translated in a variety of in vitro translational systems, and subsequently identified by selective immunoprecipitation. Translation of polyA$^+$-RNA in a cell-free reticulocyte lysate system results in the synthesis of precursors of MHC products which display the gross structural features of the MHC antigens, but not those features unique to the mature, membrane-bound MHC antigens. The generation of mature MHC products in vitro requires the presence of a membrane fraction during synthesis. This was demonstrated by translating polyA$^+$-RNA in either frog oocytes or in the reticulocyte lysate supplemented with dog pancreatic microsomes. In these cases, the observed product has acquired the unique structural features of the mature MHC antigens. Cell free translation of size fractionated mouse RNA species has confirmed that the RNA species encoding murine transplantation antigens have sedimentation coefficients of approximately 16S; further, RNA species encoding Ia antigens have been determined to be 12S.

Screening of both mouse and pig recombinant DNA libraries with the human MHC cDNA probe has identified a number of genomic fragments which contain MHC genes. These recombinant phages are presently being characterized to determine which members of each multigene family are represented in the recombinant DNA.

Significance to Biomedical Research and the Program of the Institute: Genetic studies in a number of mammals, including man, mouse and guinea pig, have demonstrated the existence of immune response genes which control cellular interactions leading to both humoral and cellular immunity. The inability of an animal to respond to a given antigen or to reject foreign tissue probably represents a genetic defect. Despite the clear importance of the major histocompatibility locus in the immune response, nothing is known at the molecular level about the content, genetic organization or regulation of expression of this multigene family. An understanding of the molecular basis of the MHC may afford the possibility of treating various immunodeficiency diseases by appropriate genetic manipulations.

Proposed Course of Project: The analysis of the organization of the genes encoding the MHC will proceed along two lines: (1) Purification of the mRNA species encoding the MHC products in both mouse and pig. Once purified, these mRNA molecules will be used as templates to synthesize double-strand cDNA, which will be propagated in recombinant plasmids. It will then be possible to analyze the various MHC RNA species found in the pig to ascertain their relationships to each other and to compare these expressed sequences with those found in the mouse.

(2) Characterization of isolated MHC genomic clones. The genomic clones already isolated will be analyzed to determine which MHC products they encode. Once this has been established, it will be possible to determine the evolutionary relationships between the pig and mouse MHC gene families. It should also be possible to begin to examine the regulation of expression of these genes.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05084-03 I

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Antibody Dependent Cellular Cytotoxicity.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Wunderlich	Senior Investigator	I NCI
OTHER:	J. Connolly	Microbiologist	I NCI
	D. Segal	Senior Investigator	I NCI
	J. Titus	Chemist	I NCI
	S. Dower	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.2	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES x ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human peripheral blood lymphocytes with augmented ADCC activity resulting from 2-day in vitro stimulation by Concanavalin A have been quantitatively assessed by flow microfluorometry for IgG Fc receptor density, affinity and distribution profiles. Analysis of 100,000 cell aliquots of Con A stimulated and control, non-stimulated cells from each of four donors revealed that 4-25% of cells bore IgG Fc receptors (FcR). Analysis of the FcR positive cells from each of the 4 donors showed that the average receptor density for a donor ranged from 65,000 to 72,000 receptors per cell, assuming that each subunit of Fc receptor ligand (dimeric anti-DNP) bound to an Fc receptor. The average binding constant per donor ranged from 1.8 to 2.6×10^7 (M^{-1}). Con A stimulation did not have a consistent effect on either of these parameters, and the changes that did occur represented less than 10% of the values for control cells. All fluorescence distribution profiles of cells with fluorescence labeled Fc receptor were unimodal and nearly overlapping for Con A stimulated and control cells.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: This project has been directed at understanding the cellular nature and mechanism of antibody dependent cell-mediated cytotoxicity (ADCC) mediated by activated human lymphocytes.

Methods Employed: Heparinized peripheral blood cells are enriched for leukocytes by sedimentation in Plasmagel and are depleted of monocytes and red blood cells by carbonyl iron ingestion followed by Ficoll-Hypaque cell separation. The cells are cultured for 1-2 days in medium with 20% allogeneic serum, during which time they are stimulated with the mitogen Concanavalin A.

Different doses of stimulated or control lymphocytes are then coincubated as effector cells for 4 hrs. with ^{51}Cr-labelled target cells, pretreated with antiserum from a multiply transfused aplastic donor. Usually a human B lymphoblastoid cell line has been used as a source of target cells. Presensitized with IgG antibodies, these target cells are highly sensitive to ADCC and provide a linear assay for effector cell activity.

The affinity and density of IgG Fc receptors on lymphocyte surfaces are determined using covalently cross linked dimers of rabbit anti-DNP antibodies. Lymphocytes treated first with different doses of dimer and then with an excess of fluorescein-conjugated affinity purified Fab^2 goat rabbit IgG are analyzed for cell fluorescence using a FACS II instrument. Dr. Segal previously demonstrated that: (1) dimers bound to cell surface Fc receptors are relatively resistent to washing procedures, (2) dimers bound to cell surface Fc receptors remain cell surface associated after washing procedures, (3) the procedures for labelling IgG dimers bound to Fc receptors with fluorescent anti-IgG antibody do not affect the amount of bound dimer, and (4) fluorescent emission is a linear function of the number of IgG dimers bound per cell.

Major Findings: Previously, we demonstrated that stimulation of human peripheral blood lymphocytes in vitro with Con A enhances effector cell activity for ADCC. Analysis of receptors for antibody (FcR) on the surface of effector cells showed no change in binding profiles (affinity) or density. Availability of more specific reagents has now permitted direct quantitation of these parameters. Analysis of 100,000-cell aliquots of Con A stimulated and control, non-stimulated cells from each of 4 donors revealed that 4-25% of cells bore IgG-Fc receptors. Analysis of the FcR positive cells from each of the 4 donors showed that the average receptor density for a donor ranged from 65,000 to 72,000 receptors/cell, assuming that each of subunit of dimeric anti-DNP bound to an Fc receptor. The average binding constant was $1.8\text{-}6.9 \times 10^7$ (M^{-1}). Con A stimulation did not have a consistent effect on either of these parameters and what changes did occur represented less than 10% of the values for control cells. All of the fluorescence distribution profiles of cells whose FcR were labelled with different concentrations of antibody dimer were unimodal, and Scatchard plots of the average antibody binding level as a function of antibody concentration were linear--both findings showing no evidence for heterogeneity in binding constants. Dimer binding profiles of Con A stimulated and control cells nearly overlapped when

compared for each donor at the different concentrations of antibody dimer. An important point of this analysis is that it was carried out at 0° C. At higher temperatures, increased membrane fluidity induced by Con A stimulation might increase the effective FcR affinity for antibody.

Significance to Biomedical Research and the Program of the Institute: Antibody dependent cellular cytotoxicity provides a mechanism whereby normal lymphoid cells can be utilized for target cell destruction. Evidence has accrued from a variety of laboratories that the mechanism may operate in human rejection of certain parasites, fungi, viruses, tumor-cells and allografts. ADCC activity is dependent on and probably proportional to the Fc receptor density on killer cells and the affinity of these receptors for immunoglobulin. Accurate quantitation of these parameters is thus important not only for comparing different types of effector cells (e.g. activated vs. non-activated) but also for gauging FcR site availability to antibody coated target cells.

Proposed Course of Project: A major technical advance is needed for significant future progress on this project: a means for purifying the sub-population of lymphocytes which destroy antibody treated target cells (K cells). Rapid advances in generation and characterization of hybridoma monoclonal antibodies and lectins which react with differentiation determinants on human lymphocytes are now occurring in other laboratories and it is hoped that these reagents will provide the means of purifying K cells. Thus, future work on this project will be delayed until the specificity patterns of these reagents are more clear. Publications for this project will be incorporated under other projects by this investigator and this project will be terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05085-03 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Role of Cell Interactions in the Development of Syngeneic Tumor Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Fujiwara	Visiting Associate	I	NCI
Other:	G. M. Shearer	Senior Investigator	I	NCI
	T. Tsuchida	Visiting Fellow	I	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This work was initiated to study the role of cell interactions in the development of syngeneic tumor immunity. It has been shown that the degree of specific anti-tumor immunity developed both in vivo and in vitro in response to TNP- conjugated tumor cells can be increased by the addition to TNP-reactive helper T cells. The cytotoxic cell in vitro has been shown to be a T-cell. The nature of the lymphocytes involved in in vivo killing is under investigation.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The aim of this project is to study the role of cell interactions in syngeneic tumor immunity. Previously, a system was explored in which animals were immunized with syngeneic tumor cells modified with additional determinants. Supplementation of helper (amplifier) T-cells specific for those determinants amplified the generation of killer T cells against native tumor antigens. Specifically, helper T-cell activity against 2,4,6-trinitrophenyl (TNP) hapten was elicited by immunizing mice with TNP-conjugated isologous mouse gamma globulin (MGG). Addition of TNP-helper T cells during the immunization of C3H/He mice with TNP-conjugated syngeneic tumor cells led to a significant enhancement of killer cell activity against the native, unmodified tumor.

These preliminary results were observed after several immunizations with TNP-conjugated tumor cells. The requirements for producing optimal augmentation of in vitro cytotoxic effector cell activity and in vivo protective immunity, as well as the nature and specificity of in vitro and in vivo effector mechanisms augmented by the above cell-cell interaction system are the subject of the current project.

Methods Employed: TNP-reactive helper T-cells were induced by intraperitoneal (i.p.) inoculation with 500 ug TNP-D-GL in saline, followed by i.p. injection of 100 ug of syngeneic TNP-MGG in complete Freund's adjuvant (CFA) 3 days later. Six weeks after the immunization with TNP-MGG, animals were inoculated i.p. with 1.5×10^7 TNP-conjugated LSTRA tumor cells at 10- to 14-day intervals. Two weeks after the final immunization with TNP-LSTRA, spleen cells were sensitized to in vitro and cytotoxic effector cells were measured by ^{51}Cr release assay in vitro and in vivo tumor-neutralization test.

Major Findings: The study indicates that the addition of TNP-reactive helper T-cells to the BALB/c-LSTRA syngeneic tumor system at the time of immunization with TNP-conjugated LSTRA tumor cells results in the accelerated development as well as the amplified generation of tumor-specific cytotoxic effector T-cells in vitro and tumor-neutralizing T-cell activities in vivo. We have also demonstrated the absolute requirement of the TNP-reactive amplifier T-cell system for the induction of a powerful in vivo immunity as observed in the Winn assay.

Significance to Biomedical Research and the Program of the Institute: The role of helper cells in immune reactions may be clarified and possible applications to the immunotherapy of tumors made possible.

Proposed Course of Project: Additional research should be performed to (1) establish a simpler and more effective condition under which strong amplifier T-cells can be induced and (2) determine the nature of the amplifier T-cells and cells which are responsible for in vivo immunity. Helper cell activity will be analyzed as a possible approach for augmenting cell-mediated immunity to tumor antigens. Helper activity will be raised by immunization

against TNP and other haptens. Such helper cells will be tested for their potential to effect more efficient immunity to tumor antigens. In these studies the B16 mouse melanoma line as well as radiation-induced lymphoid tumors will be studied both in the parental strain of origin and in F_1 hybrid strains to determine whether F_1 resistance to the tumor can be detected and manipulated by helper cells.

Publications

Fujiwara, H., and Shearer, G. M.: Suppressive effect of X-irradiated tumor cell presensitization of the induction of syngeneic tumor immunity. II. Opposite effects of intravenous administration of TNP-conjugated tumor cells on the development of anti-tumor and anti-TNP-self cytotoxic effector cells. Cellular Immunol. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 05086-03 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immune Response Gene Regulation of the Immune Response In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Hodes	Chief, Immunotherapy Section	I NCI
	P. I. Nadler	Investigator	I NCI
Other:	A. Singer	Senior Investigator	I NCI
	G. Miller	Investigator	I NCI
	D. H. Sachs	Chief, Transplantation Biology Section	I NCI
	Y. Asano	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The cellular expression of immune response (Ir) gene function was studied in an in vitro system of primary antibody responses to the TNP conjugates of (T,G)-A--L and (H,G)-A--L. These responses require the participation of T cells and accessory cells as well as B cells, and are under the control of genes mapped to the K or I-A subregions of the H-2 complex. It was demonstrated that the function of accessory cells in responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L is under the control of genes which also map to K or I-A. In contrast, both B cells and T cells from nonresponder H-2^a strains to TNP-(T,G)-A--L are competent in supporting primary antibody responses to this antigen, and therefore do not appear to express the Ir gene defect present in these strains. Subsequently, in vitro augmented primary responses to TNP-nuclease (TNP-NASE) have been established and documented to be under the control of H-2 linked Ir gene(s). For these responses as well, accessory cell function was shown to be under Ir gene control. Through the use of intra-H-2 recombinant strains, the Ir gene(s) controlling responsiveness to TNP-NASE were shown to map to I-B.

Project Description

Objectives: The major objective of this project is to investigate the mechanism of genetic regulation of antibody responses. Initial studies identified the cellular level of Ir gene expression for the in vitro responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L. Currently ongoing studies are directed at characterizing the Ir gene control of responses to TNP-NASE, including the possible function of gene complementation in this control.

Methods Employed: The methods employed have been described in detail. See project No. Z01 CB 05064-04 I.

Major Findings: Background work has demonstrated that primary or augmented primary in vitro antibody responses could be generated to a number of soluble TNP conjugates of protein or polypeptide antigens. These responses are both T cell-dependent and accessory cell-dependent. The in vitro primary IgM responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L are under strict H-2 linked gene control by genes mapping to the K or I-A subregions. Accessory cell function is under H-2 linked Ir gene control, mapping to K or I-A, while neither B cells nor T cells of the H-2^a haplotype express detectable Ir gene defects for the response to TNP-(T,G)-A--L.

An in vitro system was established in which TNP-specific responses to TNP-conjugated Staphylococcal nuclease (TNP-NASE) were generated by spleen cells from NASE-primed mice. These responses were T-cell and accessory cell-dependent, and under H-2-linked Ir gene control, with strains of the H-2^a haplotype being responders and H-2^b strains nonresponders. Ir gene control mapped to I-B and was not explained by complementing genes in I-A and I-E/C. Cell fractionation experiments have shown that accessory cell function is under Ir gene control for the response to TNP-NASE. Experiments carried out with a hybridoma anti I-A^k reagent have demonstrated that this reagent is capable of inhibiting the response of (H-2^a x H-2^b)F_1 spleen cells to TNP-NASE. These findings suggest that a gene (or genes) in I-A, as well as genes in I-B may regulate the response to TNP-NASE.

Significance to Biomedical Research and the Program of the Institute: Genetic control of immune responses has been demonstrated in widely studied systems, including those responses to biologically "natural" antigens including allergens, viral determinants, and tumor antigens. In order to understand the mechanism of differentially reactivity and susceptibility to these natural stimuli, the mechanism of Ir gene regulation of responses to defined stimuli may provide informative insights.

Proposed Course of Project: Conventional and monoclonal anti-Ia antibodies will be used to probe for the I region products which function in Ir gene expression.

Publications

Dickler, H. B., Cowing, C., Ahmann, G. B., Hathcock, K. S., Sachs, D. H., Hodes, R. J. and Singer A.: Expression of Ir genes and Ia antigens by adherent accessory cells required for antigen-specific antibody forming cell responses. In van Furth R. (Ed.): Mononuclear Phagocytes. Functional Aspects, Part II. The Hague, Martinus Nijhoff B.V.-, 1980, pp. 1909-1922.

Nadler, P. I., Miller, G. P., Sachs, D. H., and Hodes, R. J.: Ir gene control of in vitro antibody responses to TNP-nucelase. J. Immunol. in press.

Singer, A., Hathcock, K. S., and Hodes, R. J.: Self-recognition in allogeneic chimeras. A radiation-resistant host element dictates the self specificity and immune response gene phenotype of T-helper cells. J. Exp. Med., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05087-03 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

T Cell Recognition in the Mixed Lymphocyte Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Hodes	Chief, Immunotherapy Section	I NCI
	R. Gress	Clinical Associate	I NCI
	P. Nadler	Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1	.25

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ability of subpopulations of murine spleen cells to stimulate a mixed lymphocyte response (MLR) was studied. It was found that T cells and B cells were poor stimulators of an MLR across H-2 or Mls differences, while non-T, radiation resistant Ia positive splenic adherent cell (SAC) were 20-50 times more efficient as stimulators of these MLR's than unseparated spleen cells. These results suggest that Ia^+SAC may be the predominant stimulating cells in spleen cell populations, and the preferential target for T cell recognition in cell interaction events. It has further been demonstrated that T cells responding to Mls determinants recognize such determinants in the context of "self" H-2 determinants. Studies employing K region mutants have investiated the influence of chimeric maturation environment upon the alloreactive T cell repertoire to mutant determinants, and suggest that this alloreactive repertoire is in fact influenced by the environment in which T cells mature.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Since the MLR is a model for T cell recognition of cell surface determinants, the objective of these studies is to characterize T cell recognition of allogeneic determinants.

Methods Employed: Purified T cells (nylon non-adherent spleen cells) purified B cells (G10 Sephadex passed and RAMB + C treated) and splenic adherent cells (glass adherent, non-T radiation resistant spleen cells) (SAC) were prepared and compared for their ability to stimulate a proliferative response by allogenic whole spleen cells. Stimulator SAC populations are further evaluated by 1) treatment with anti-Ia reagents and C, 2) addition of monoclonal anti-Ia and anti-H-2 antibodies directly to the culture, and 3) fractionation of SAC on the basis of their ability to phagocytise latex particles. In vitro generation of cytotoxic T lymphocytes (CTL) was carried out by MLR cultures.

Major Findings: A non-T radiation resistant spleen adherent cell population (SAC) was up to 20-50 times more efficient in stimulating MLR on a per cell basis than an unseparated spleen cell population; and these SAC express Ia determinants encoded by genes in I-A and I-E/C. These findings were observed both for MLR to H-2-differences and for MLR to Mls stimulating determinants.

Mls encoded determinants appear to be unique in that they are the only non-MHC determinants capable of stimulating primary MLR. Studies were undertaken to determine whether responding T cells recognize Mls product alone, or recognize Mls in the context of H-2. Limiting dilution conditions were established under which the magnitude of MLR proliferative response to Mls determinants was proportional to the number of responding T cells. Experiments carried out under such conditions demonstrated that T cells do not respond to Mls determinants alone, but rather that distinct T cell subpopulations exist which recognize Mls in the context of "self" H-2 determinants.

CTL were generated by the response of B6 ($H\text{-}2^b$) spleen cells against stimulating cells differeing from B6 only by point mutations in the K^b region. These responses were compared with the responses of $H\text{-}2^b \rightarrow H\text{-}2^d$ or $H\text{-}2^d \rightarrow H\text{-}2^b$ chimeras to K^b mutant cells or entirely allogeneic stimulators.

The responses to selected K^b mutants were strictly determined by the environment in which responding T cells had matured, so that normal $H\text{-}2^b$ or $H\text{-}2^d \rightarrow H\text{-}2^b$ chimeric cells generated strong CTL responses, while $H\text{-}2^b \rightarrow H\text{-}2^d$ responding cells were selectively unresponsive.

Significance to Biomedical Research and the Program of the Institute: The mixed lymphocyte response provides a useful model for T cell recognition as well as an in vitro correlate of allograft rejection. Further understanding of the recognition process as well as the primary stimulator cell should provide insight into controlling or preventing allograft and/or tumor challenges.

Proposed Course of Project: Specific anti-H-2 and anti-Ia reagents purified from hybridoma cell lines are being evaluated for their ability to block MLR responses to either H-2 or Mls differences. In addition, further work is in progress to study the mechanism by which host environment determines the alloreactive T cell repertoire.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05088-03 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effects of Graft Vs. Host Reactions on Cell-Mediated Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
Other:	U. Hurtenbach	Visiting Associate	I NCI
	J. Chalmer	Visiting Fellow	I NCI
	T. Tsuchida	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The intravenous injection of F_1 hybrid mice with parental spleen cells resulted in a loss in the ability of the F_1 mice to generate T-cell mediated cytotoxic responses in vitro to TNP-self and alloantigens. The loss of response potential depended on the H-2 type of the parental cells, since H-$2^{k,a}$ spleen cells induced unresponsiveness, whereas H-2^b spleen cells did not. The phenomenon is: (a) strain-specific (possibly depending on the C57Bl/10 genetic background), since strains of mice with other genetic backgrounds injected with parental cells do not exhibit the loss of immune potential; and (b) dependent on a reaction against F_1 alloantigens by grafted parental cells (GVH), since loss of immune activity was associated with enlarged F_1 host spleens. Suppressor cells were found to be responsible for loss of immune potential. The failure of lymphocytes from parental strains was shown to be due to F_1 resistance to parental T cells, which mapped to H-$2D^b$. Protection against GVH-associated suppression could be achieved using anti-H-2 sera directed against specificities of donor or host.

Project Description

Objectives: The purpose of this project is to investigate the phenomenon of immunosuppression induced by or associated with a graft vs. host reaction, the immunogenetics associated with the resistance in some strain combinations of the graft vs. host associated suppression, and to attempt to establish whether this immunosuppression is correlated with autoimmune or neoplastic states following a graft vs. host reaction.

Methods Employed: F_1 hybrid mice of various strain were injected intravenously with from 1 to $40x10^6$ F_1, parental, or allogeneic spleen cells. At various times after injection, the spleens of the injected F_1 mice were sensitized in vitro against: (a) parental or F_1 syngeneic cells modified with TNBS; or (b) allogeneic spleen cells. The effector cell actively generated 5 days later was tested on the appropriate ^{51}Cr-labelled target cells. Mapping studies were performed using inbred and recombinant mice on the C57BL/10 genetic background, as well as strains of other genetic backgrounds. Mice were injected with anti-sera or monoclonal reagents specific for H-2 region and subregion gene products. These reagents were supplied by the laboratory of Dr. D. H. Sachs.

Major Findings: F_1 hybrid mice on the C57BL/10 genetic background injected intravenously with viable parental spleen cells lost their ability to respond by in vitro generated cytotoxic reactions to TNP-self and alloantigens. The loss of cytotoxic potential was detected as early as four days after injection and persisted for at least 30 days after injection. Recovery from immunosuppression was detected 40-45 days after injection of parental cells. The abolition of cytotoxic potential: (a) appeared to depend on a graft vs. host reaction by parental lymphocytes against host alloantigens; and (b) was dependent on the H-2 haplotype of the parental cells used, since the injection of B10.A or B10.BR but not C57BL/10 (B10) parental cells resulted in loss of immune reactivity. The latter observation indicated that the B10 parental cells injected were rejected by the F_1 anti-parent reaction known as hybrid histoincompatibility (Hh). Genetic studies indicate that the $H\text{-}2^b$ homozygous determinant recognized by the F_1 maps to the H-2D region, which is compatible with an Hh-like phenomenon. The abolition of cytotoxic potential by the GVH reaction is the result of an active suppressive mechanism, since the addition of spleen cells from parental-injected F_1 mice to normal F_1 spleen cells led to the inactivation of the cytotoxic potential of the normal cells. The GVH associated immunosuppression may strain dependent, and may require non-H-2-linked genetic factors associated with the C57BL background, since mouse strains not on the C57Bl background injected with parental spleen cells did not result in suppressed immune potential. Protection against suppression was observed in F_1 mice injected with anti-H-2 antibodies specific for K, I, or D region gene products expressed either by the F_1 host or parental donor. Such protection was observed: (a) by using either anti-H-2 sera or monoclonal reagents, and (b) by injecting either F_1 host or parental donors. It was also found that the induction of suppression requires recognition of I region determinants expressed by the F_1 by the parental spleen cells.

Significance to Biomedical Research and the Program of the Institute: The graft vs. host (GVH) reaction and possibly Hh-type reactions are important complicating factors which affect the success of hemopoietic transplantation. Furthermore, persisting GVH reactions may be associated with autoimmune disease and the development of tumors. The observations: (a) that GVH reactions can be elicited with low numbers of lymphocytes in immunocompetent adult mice (previous reports have been limited to the demonstration of GVH in neonates or immunosuppressed animals); (b) that these GVH reactions lead to severely impaired T-cells immune functions; and (c) that such GVH reactions can be overcome by host resistance mechanisms are potentially of fundamental relevance in: (1) understanding the possible complications resulting from hemopoietic grafting; (2) investigating the significance of a GVH-induced suppressed immune system in the development of autoimmune and neoplastic disease; and (3) understanding natural resistance systems as they may be relevant in surveillance against disease and neoplasms.

Proposed Course of Project: We shall continue to investigate all aspects of the phenomenon including: (a) the genetics of the F_1 and parental cells involved; (b) the determinants recognized on the F_1 cells; (c) the mechanistic aspects of both the GVH and the suspected Hh component involved; (d) other immune functions which may be impaired including antibody production, delayed hypersensitivity, skin graft rejection, T-cell proliferative responses, and natural killer cell activity; (e) the long-term effects of the GVH including survival and the development of autoimmune disease and tumors; (f) whether certain combinations of partially allogeneic (instead of F_1 and parental) cells and hosts can lead to GVH-associated immunosuppression; (g) whether the GVH reaction is actually a component of the impaired immune state; (h) whether haplotype-specific anti-T-cell receptor suppression can be induced in the F_1 parent combination; and (i) analysis of different populations of cells involved in the induction of and protection by antibody against GVH-associated immunosuppression.

Publications

Shearer, G. M. and Polisson, R. P.: Mutual recognition of parent and F_1 lymphocytes: Selective abrogation of cytotoxic potential of F_1 lymphocytes by parental lymphocytes. J. Exp. Med. 151: 20-31, 1980.

Shearer, G. M., Polisson, R. P., Miller, M. W., and Cudkowicz, E.: Genetic control of natural resistance to graft versus host-associated suppression to T cell-mediated lympholysis. In Skamene, E., Kongshavn, P. and Landy, M. (Eds.): Genetic Control of Natural Resistance to Infections and Malignancy. New York, Academic Press. pp. 485-494, 1980.

Polisson, R. P. and Shearer, G. M.: Mutual recognition of parent and F_1 lymphocytes. II. Analysis of graft versus host-induced suppressor cell activity for T-cell mediated lympholysis to trinitrphenyl-self and alloantigens. J. Immunol. 125: 1855-1861, 1980.

Shearer, G. M. and Polisson, R. P.: Mutual recognition of parental and F_1 lymphocytes. III. Parental determinants recognized by F_1 host mice in resistance to graft-versus-host-associated immunosuppression map to H-2D^b. J. Immunol. 126: 545-547, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05090-03 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Role of Accessory Cells in B Cell Activation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Morrissey	IPA Investigator	I NCI
Other:	A. Singer	Senior Investigator	I NCI

COOPERATING UNITS (if any) I. Scher, Department of Medicine, Uniformed Services School of Medicine and Department of Experimental Pathology, Naval Institute of Medical Research, Bethesda, MD and A. Ahmed, Merck Institute for Therapeutic Research, Rahway, NJ

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A novel insight into the role of macrophage accessory cells in B cell activation was provided by the observation that the immune response to the thymic independent (T-I) antigen, TNP-Ficoll, was dependent upon the presence and function of splenic adherent accessory cells. The functional spleen adherent cell was identified as a phagocytic macrophage-like cell which bears the I-A gene product. The accessory cell for this T-I TNP-Ficoll response functioned to present antigen to B cells. This identifiable macrophage-B cell interaction was found to be obligately restricted to the Lyb 5+ B cell subset which appears late in normal B cell ontogenetic development and which is absent in hybrid mice which have received the X-linked immune deficiency gene of the CBA/N mouse. The existence of this important cell interaction required for activation of B cells in response to accessory cell dependent antigens such as TNP-Ficoll (T-I) and the thymic dependent (T-D) TNP-KLH provides explanation for the immune response deficit to the T-I and T-D antigens in mice which bear the X-linked CBA/N genetic defect.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The major objectives of this project are: 1) to identify the specific immune function and the cell surface phenotype of the macrophage-like accessory cell important for immune responses to thymic dependent and thymic independent antigens; 2) to investigate the mechanism of B cell activation mediated by direct interaction of B cells with antigen presenting accessory cells.

Methods Employed: Spleen cells from normal or immune defective mice are dispersed into single cell suspensions and placed into microculture with TNP-modified antigens (KLH, Brucella abortus, Ficoll) for 4 days. The cells are then harvested and assayed for IgM anti-TNP antibody forming cells by the slide modification of the Jerne plaque technique.

Splenic B cells are obtained by treating spleen cells with a T-cell specific rabbit anti-mouse brain and complement. Subpopulations of B cells are obtained based on their expression of a non H-2 encoded cell surface antigen, Lyb 5. CBA/N B cells constitute a pure population of immature B cells which lack the marker Lyb5. Treatment of normal B cells with an alloantiserum, anti-Lyb 5 and complement results in deletion of the mature Lyb 5 positive population, thus creating the CBA/N defect in normal spleen cells. Splenic T cells are obtained by passage over a nylon wool column. Splenic adherent cells are obtained by allowing spleen cells to adhere to glass petri dishes for 2 hours, discarding the nonadherent cells, and finally collecting the adherent cells by the addition of EDTA. Spleen cells are depleted of macrophages by passage over a G10 sephadex column. In some experiments the ability of splenic macrophages to phagocytose fluorescent latex beads was utilized to allow purification of phagocytic and non-phagocytic populations using the fluorescence activated cell sorter (FACS).

Major Findings: The in vitro response to TNP-Ficoll, a polysaccharide T-I antigen was dependent upon the presence of adherent accessory cells since passage of spleen cells over G-10 Sephadex columns prior to culture abolished the response. The required accessory cell was both adherent and phagocytic since responses depleted by either spleen cell passage over G-10 Sephadex or by removal of cells phagocytosing fluorescent latex on the FACS, could be fully reconstituted both by addition of splenic adherent cells or by the phagocytic fraction of adherent cells. In addition, SAC could be pulsed with TNP-Ficoll to present the antigen to activate B cells to form antibody. Treatment of normal spleen cell populations with anti Lyb5, like passage of cells over G10 Sephadex, abrogated TNP-Ficoll (T-I) and TNP-KLH (T-D) responses. This effect was not a result of the cytotoxic deletion of macrophages but, rather, of the deletion of B cells. Thus, the macrophage functioning in T-I, as well as T-D responses has the phenotype Ia^+, Lyb 5^-. The fact that responses requiring adherent accessory cells, such as the T-D TNP-KLH and the T-I TNP-Ficoll responses, emanate entirely from Lyb 5^+ B cells, suggested that only Lyb 5^+ B cells respond to macrophage activation signals. This possibility was validated by the demonstration that TNP-BA, a T-I antigen which activates both Lyb 5^- and Lyb 5^+ B cells when added directly to culture, activated

only Lyb 5^+ B cells when presented by pulsed macrophages. This concept of restricted activation of Lyb 5^+ B cells by macrophages provides important insight into the X-linked immune deficiency of the CBA/N mouse because this mouse lacks the Lyb 5^+ B cell subset and exhibits immune deficiency to macrophage dependent antigens.

Significance to Biomedical Research and the Program of the Institute: Utilization of techniques for separation of splenic lymphocyte and macrophage populations has facilitated precise understanding of the cellular interactions involved in the normal immune response, and the abnormalities of these interactions in a genetic immune deficiency. Such understanding is the primary step to allow for the possibility of alteration of imbalanced immunity as it appears in clinical medicine.

Proposed Course of Project: Further work will be directed to the delineation of of the nature of the signals transmitted from macrophages to T and B lymphocytes populations to effect an immune response.

Publications

Boswell, H. S., Ahmed, A., Scher, I., and Singer, A.: Role of accessory cells in B cell activation. II. The interaction of B cells with accessory cells results in the exclusive activation of an Lyb5+ B cell subpopulation. J. Immunol. 128: 1340-1348, 1980.

Boswell, H. S., Nerenberg, M. I., Scher, I., and Singer, A.: Role of accessory cells in B cell activation. III. Cellular analysis of primary immune response deficits in CBA/N mice: Presence of an accessory cell-B cell interaction defect. J. Exp. Med. 152: 1194-1209, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05091-03 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Target Antigen Recognition by Cytotoxic T Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.A. Henkart	Senior Investigator	I NCI
Other:	H. Pehemberger	Fogarty Fellow	I NCI
	G. M. Shearer	Senior Investigator	I NCI
	R. B. Levy	Senior Staff Fellow	I NCI
	K. Ozato	Visiting Associate	I NCI
	D. H. Sachs	Acting Chief	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.8	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1)Plasma membrane vesicles were prepared from tumor cells which are good target cells for cytotoxic T lymphocytes (CTL); by comparing their interaction with CTL before and after fusion of the vesicle membrane with the membrane of a tumor cell not recognized by the CTL, it was concluded that insertion of the H-2 antigen recognized into a living cell membrane greatly enhances recognition by CTL. (2)H-2 antigens on lymphoid cells were studied in binding experiments with labeled monoclonal anti-H-2. One example of a single H-2 antigen having two distinct determinants was found. (3) Spleen cells modified by several haptenic -SH reagents were found to stimulate the production of H-2 restricted hapten-specific CTL after in vitro culture with syngeneic spleen cells. Comparison with previously developed hapten modified CTL recognition systems shows the new system to be more H-2 restricted; the recognized hapten is on an -SH group not part of H-2. Epidermal cells modified with these reagents could also stimulate such CTL.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To understand the molecular mechanisms involved in the binding of cytotoxic T lymphocytes to the target cells which they specifically recognize. This is being attempted by several approaches: (1) To develop a means of quantitatively studying the binding reaction without looking at cytotoxicity; (2) To better understand the qualitiative and quantitative display of transplantation antigens on the membrane of a variety of cells, since these antigens apear intimately involved in the recognition of all types of target cells by CTL; (3) To develop new and better defined systems for H-2 restricted killing of hapten-modified target cells.

Methods Employed: (1) Plasma membrane vesicles are prepared from RDM-4 tumor cells ($H\text{-}2^k$) by the nitrogen decompression method. Such vesicles were adhered to P388D1 ($H\text{-}2^d$) tumor cells by wheat germ agglutinin, and in some cases fused with 40% polyethylene glycol. Alloreactive cytotoxic T lymphocytes are obtained from secondary in vitro cultures of $H\text{-}2^b \times H\text{-}2^d$ spleen cells with irradiated $H\text{-}2^k$ spleen cells. (2) Purified monoclonal anti-H-2 antibodies are labeled with ^{125}I and allowed to bind to several million spleen cells. Bound antibody is detected by spinning the cells through oil and counting them in a gamma counter. (3) The most utilized reagent for modifying cell surface SH groups is 1,5-iodoacetylethylenediaminenapthylene sulfonic acid (I-AEDANS). The cells are modified by treatment with 1mM reagent, pH 8, 37° for 30 minutes. Standard CML culture conditions are used to generate CTL, except that cultures are carried out for 7 days, and $H\text{-}2^b$ is the high responder. ^{51}Cr labeled target cells are modified by the above procedure. Anti-AEDANS was made in rabbits by injection of a KLH conjugate. Affinity-purified anti-AEDANS was FITC labelled and used with the FACS to quantitate cell-surface hapten groups. That the CTL recognize AEDANS on -SH groups was shown by pre-treatment of the target cells with other SH reagents prior to the AEDANS reaction. That H-2 is not the recognized AEDANS modification was shown by co-capping and the use of H-2 mutants. Epidermal cells are prepared by dissecting off the body skin of appropriate mice and dissociating the cells using trypsin.

Major Findings: (1) Our studies have established that recognition of membrane vesicles as such by CTL is inefficient. If vesicles are bound to the surface of ^{51}Cr labelled target cells not bearing antigens recognized by the CTL, no target cell lysis occurs. However, after fusion of the vesicle membranes into the target cell membrane with PEG, these cells are lysed by the CTL. Thus some aspect of the living cell membrane confers "recognizability" to the H-2 antigens. (2) Labelled monoclonal anti-$H\text{-}2K^k$ antibodies were shown to bind specifically to living cell membranes. Such binding was shown to be inhibitable with cold anti-$H\text{-}2K^k$ antibodies, and one combination of two monoclonal antibodies were found to bind non-competitively. This was interpreted as showing two spatially separate sites on the antigen recognized by specific antibodies. (3) Cell surface -SH groups were modified with haptenic -SH reagents; such cells stimulated the production of H-2 restricted, hapten specific CTL when cultured in vitro with syngeneic spleen cells. It was shown that CTL recognize modified -SH groups on the membrane which are not on H-2, there are about 10^4 haptens/cell, and that recognition can be

specifically blocked by anti-hapten antibody. This "modified-self" system is significantly more H-2 restricted than lysine-reacting haptens like TNP. In an effort to detect CTL restricted by hapten-modified differentiation antigens, epidermal cells modified with such haptens were found to induce CTL when used as stimulator cells in the in vitro culture; in vivo priming of the responder cells was required. Thus far, no epidermal-specific component of the recognition has been detected, since both lymphoid and epidermal cells are lysed by these CTL.

Significance to Biomedical Research and the Program of the Institute: T lymphocytes play a central role in most immune responses and much recent speculation about their antigen receptors has resulted from inferences from complex functional experiments. We hope to devise ways of studying this recognition process by carrying out more chemically defined studies of the binding process. An understanding of this receptor's interactions will not only aid in a basic understanding of the process of CTL killing, but also serve as a model for all T-cell-cell interactions in the immune system.

Proposed Course of the Project: Methods are being tried out to improve the efficiency of membrane fusion. We are currently carrying out quantitative analyses of H-2 antigens on cell membranes. Methods are being devised to short-circuit the normal recognition receptors using chemically defined cross-linking between CTL and target cell. Cloned CTL will be used to eliminate the heterogeneity in the currently used populations.

Publications

Levy, R.B., Shearer, G.M., Richardson, J.C., and Henkart, P.A.: Cell mediated lympholytic responses against autologous cells modified with haptenic sulfhydryl reagents. I. Effector cells can recognize two distinct classes of hapten-reactive self sites on cell surface proteins. J. Immunol., in press.

Levy, R.B., Henkart, P.A., and Shearer, G.M.: Cell mediated lympholytic responses against autologous cells modified with haptenic sulfhydryl reagents. II. Analysis of the genetic control of cytotoxic responses to sulfhydryl and amino reactive reagents. J. Immunol., in press.

Ozato, K., Henkart, P.A., Jansen, C., and Sachs, D.H.: Spatially distinct allodeterminants of the H-2K^k molecule as detected by monoclonal anti-H-2 antibodies. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05092-02 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Cellular Expression of Idiotypic Markers and Role in Immune Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.I. Nadler	Investigator	I NCI
Others:	G. Miller	Investigator	I NCI
	D.H. Sachs	Chief, Transplantation Biology Section	I NCI
	R. J. Hodes	Chief, Immunotherapy Section	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION Immunotherapy and Transplantation Biology Sections

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The antibody and T cell proliferative responses to Staphylococcal nuclease, a small peptide antigen, have been shown to be under Ir gene control. In addition anti-idiotypic antisera have been produced with specificity for anti-nuclease antibodies of a number of mouse strains. It has been demonstrated that these idiotypic markers are linked to allotype markers but not to MHC encoded (H-2) determinants. The present study has investigated the cellular expression of idiotypic markers in this Ir gene controlled response. In vitro augmented primary responses to TNP-nuclease could be generated after priming with nuclease (antigen), anti-idiotypic antisera (Ab2), or anti-[anti-(anti-idiotype)] (Ab4). These responses to TNP-Nase were shown to be inhibited by anti-idiotype present in culture. It was demonstrated that antigen, anti-idiotype or Ab4 primed T cells were required and that treatment of the primed T cells with anti-idiotype and complement abrogated their helper cell activity. Thus, direct evidence for helper T cell expression of idiotypic markers has been obtained although it is as yet unclear whether the T cell synthesizes or acquires these cell surface determinants. Studies with nuclease specific T cell clones are under way to further elucidate the nature of helper T cells and their antigen-specific receptors.

Project Description

Objectives: The expression of V_H region markers (idiotypes) on sIg molecules of B cells is well established. There has been considerable controversy regarding the existence of similar V_H encoded idiotypic determinants on T cell subpopulations. The present studies were undertaken to examine the expression of idiotypic markers on T cells participating in vitro antibody responses to TNP-Nuclease.

Methods Employed: An in vitro augmented primary response to TNP-Nuclease was developed in which accessory cells, unprimed B cells, and T cells primed with Nuclease (Ag), anti-nuclease anti-idiotype (Ab2) or anti-[anti-(anti-idiotype)] (Ab4) were required. IgM plaque forming cell responses to TNP-Nuclease are antigen-dependent and TNP-specific. Standard procedures for preparation of B cells (G-10 Sephadex passage and RAMB+C treatment), T cells (nylon column passage), accessory cells (glass adherence, irradiation, and RAMB+C treatment), and accessory cell depleted spleen cells (G-10 Sephadex passage) were employed. Anti-idiotypic antisera were prepared and purified as described in Project No. Z01-CB-05036-08 I. Functional competence of spleen cell subpopulations after treatment with anti-idiotypic antisera + C was assessed. An ELISA (enzyme linked immunoadsorbent assay) was developed to measure anti-nuclease antibodies in serum and/or culture supernatants. This assay was also adapted for detection of anti-idiotypic antibodies which inhibit anti-Nase antibody binding to antigen (Nase) coated plates, and for detection of Nase idiotype in serum by inhibition of anti-idiotype binding to idiotype coated plates.

Major Findings: (1) In vitro TNP-specific IgM augmented primary responses to TNP-nuclease could be generated after priming spleen cells with either nuclease (Ag), anti-nuclease anti-idiotype (Ab2) or anti-[anti-(anti-idiotype)] (Ab4). These responses required primed T cells, B cells, and accessory cells.

(2) This in vitro augmented response to TNP-nuclease was shown to be under Ir gene control paralleing the control of the in vitro T cell proliferative response to nuclease and the in vivo antibody response to nuclease (see Project No. Z01-CB-05036-08 I and Z01-CB-05064-04 I). Accessory cells for these in vitro responses to TNP-Nase were shown to express the Ir gene defect.

(3) In vitro PFC responses to TNP-nuclease of nuclease, anti-idiotype (Ab2) or Ab4 primed BALB/c spleen cells were inhibitable by the presence in culture of anti-idiotypic antisera specific for a pool of BALB/c anti-Nase antibodies but not by anti-idiotypic antisera to SJL anti-Nase or normal pig immunoglobulin. Cell fractionation and mixing experiments have demonstrated that this inhibition occurs at the level of helper T cells.

(4) Treatment of nuclease, anti-idiotype, or Ab4 primed splenic T cells with specific anti-idiotype + C markedly diminished or abrogated the ability of these cells to provide helper cell function for in vitro responses to TNP-nuclease. Treatment of Nuclease primed B10.D2 spleen cells with anti-BALB/c anti-idiotype + C had little or no effect on their helper cell function. In addition, treatment of nuclease primed BALB/c T cells with either specific anti-SJL anti-idiotype or pig anti-BALB/c Ig+C did not affect T_H function for anti-TNP-nuclease responses.

(5) Priming of congenic high and low responder A/J and A.BY with anti-idiotype resulted in the development of idiotype-bearing, non-antigen binding molecules (ID') in both but did not circumvent the Ir gene defect of the $H\text{-}2^b$ mice for in vitro response to TNP-Nase.

(6) Treatment of B10.D2 ($H\text{-}2^d$, $Igh\text{-}C^b$) mice with either Nase or anti-BALB/c ID resulted in priming for in vitro responses to TNP-Nase. These B10.D2 mice primed with Nase expressed idiotypes normally expressed on B10.D2 antibodies in response to Nase on their helper T cells. Anti-BALB/c ID primed B10.D2 helper T cells expressed BALB/c ID determinants on their surface.

Significance to Biomedical Research and the Program of the Institute: The development of a new system in which the cellular expression of idiotypic determinants may be investigated for a response which is also under Ir gene control may be useful in investigating both the mechanism of Ir gene function and the regulation of immune responses by the proposed idiotype- anti-idiotype network. The major thrust of this endeavor has been aimed at delination of the T cell antigen receptor and its relation to the more well-characterized B cell receptor, surface immunoglobulin. Anti-idiotypic antisera recognizing determinants (V_H region encoded) linked to allotype have been used both to activate T cells in vivo for in vitro TNP-Nuclease responses and to determine whether the T helper cells required for in vitro TNP-nuclease responses bear idiotype. These demonstrations of idiotypic determinants on helper T cells similar or identical to B cell determinants should permit further characterization of the antigen receptor of T cells both functionally and chemically.

Proposed Course of Project: Studies are underway utilizing clones of nuclease primed T cells to asssess these cells for T helper cell function and expression of idiotype. In addition, attempts will be made to assess whether these idiotype-bearing T_H cells are MHC restricted or whether idiotypic restrictions exist within this system.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05093-02 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Environmental Influences on Self-Tolerance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.J. Morrissey	IPA Investigator	I NCI
Other:	A Singer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The influence of the prethymic compartment on the acquisition of tolerance to major histocompatibility antigens in the mouse was evaluated by altering this prethymic environment. Thus, strain A bone marrow cells were injected into a lethally irradiated $(AxB)F_1$ mouse. At a later time, the bone marrow from this $(AxB)F_1$ mouse was isolated and re-introduced into a lethally irradiated strain A host. Under conditions which are not now completely understood temporary tolerance to the B haplotype was achieved. To further investigate, the influence of the prethymic environment on the acquisition of tolerance to MHC antigens, $(AxB)F_1$ mice were thymectomized then grafted with a strain A thymus and later irradiated and reconstituted with strain A bone marrow. The engrafted thymus was later tested for the presence of cells bearing the B-haplotype and for reactivity toward this determinant. Preliminary results indicate that although the B haplotype is not present in the thymus, tolerance exists. Therefore, alloreactive patterns in the thymus may be affected by environmental manipulation of the pre-thymic compartment.

Project Description

Objective: These experiments will study the role of different cellular compartments on the development of tolerance and self recognition in chimeric mice.

Methods Employed: Young adult mice, strain (AxB)F_1 were thymectomized and later grafted subcutaneously with thymus lobes obtained from a newborn strain A mouse. Three to five days later, these mice were lethally irradiated and reconstituted with T-cell depleted bone marrow from strain A mice. Starting four weeks later these mice were sequentially studied at weekly intervals. The grafted thymus was isolated and the cells analyzed on the FACS II for the presence of the B haplotype. These cells were also tested for their alloreactivity and self-recognition patterns by the in vitro generation of cytotoxic lymphocytes and proliferation in a mixed lymphocyte reaction.

Major Findings: In thymectomized mice of strain (AxB)F_1 which have been grafted with a strain A thymus, irradiated, and given strain strain A bone marrow, tolerance was found to the B haplotype in the thymus in the absence of any cells bearing that haplotype. Thus, in a situation in which alloreactivity to B would be expected to arise (strain A thymocytes in a strain A thymus) it does not. Therefore the tolerizing influence must occur earlier than the thymus and also prethymocytes must express receptors for antigens prior to entry in the thymus.

The patterns of self-recognition in these mice are also being evaluated using TNP modified self as a stimulator for the generation of cytotoxic lymphocytes. Preliminary results indicate that recognition of TNP modified cells of the B-haplotype exists, but it is not yet known if this observation is a result of the degeneracy of the anti-A TNP response or actually represents restricted self recognition.

Significance to Biomedical Research and the Program of the Institute: Insights into the mechanism of self tolerance are important for our understanding of the function immune system and the generaton of the T-cell repertoire. As the underlying principles become unraveled, it is hoped that they will have a significant impact on human transplantation medicine and the immunological approach to treating cancer.

Proposed Course of Project: The project will continue to investigate the role of the bone marrow environment in self tolerance in depth. Also, the relationship between tolerance and self recognition in these chimeras will also be investigated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05094-02 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Role of the Thymus in Generation of the Self-MHC Specific T Cell Repertoire

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Kruisbeek	Guest Worker	I NCI
Other:	A. Singer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to assess the role of the thymus in the acquisition of the T cell self-MHC specific repertoire. The MHC specificity of cytotoxic precusor T cells differentiating within thymuses engrafted into athymic nude mice was determined. The results demonstrated that cytotoxic T lymphocytes from such engrafted thymuses recognized TNP in association with thymic MHC determinants in various haplotype combinations of thymus graft and nude host. In contrast, cytotoxic T lymphocytes from the spleens of these same thymus grafted nude mice recognized TNP on both nude host and thymic MHC determinants. These results are consistent with the thymus being one site which determines MHC restrictions of T cell self recognition, while there exist other factors in this experimental model which have determined the host-restricted MHC specific repertoire observed in the spleens of thymus engrafted nude mice.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The objective of this project is to assess the presumed role of the thymus in determining the recognition specificities of killer T cells directly by measuring the killer T cell responses to foreign antigens which are recognized in the context of H-2 within the thymus, rather than in the periphery.

Methods Employed: Thymocytes generally give low killer T cell responses to alloantigens and TNP-modified self H-2 antigens. Application of T cell growth factor within the cultures however allows thymocytes to express strong killer T cell responses with maintenance of specificity for the original stimulating signal. Thus, a method is available to study the specificity repertoire of thymocyte T killer cells. Complicating allogeneic effects are avoided by using radiation bone marrow chimeras and thymus-engrafted nude mice.

Major Findings: In both radiation bone marrow chimeras and thymus-engrafted nude mice, thymocyte T killer cells display recognition of TNP in association with thymic MHC determinants only. These results indicate that the recognition pattern observed in the thymus is determined by the MHC phenotype of the thymus. In fully allogeneic nude host-thymus graft haplotype combinations, such a thymus-determined recognition pattern is also observed in the spleen; however, in addition to self-recognition of thymic MHC determinants, host MHC determinants are also used as self-recognition elements. This suggests that in this model, 2 different sites, i.e., the engrafted thymus and an unknown host element, have been operational in determining the self-MHC specific repertoire of splenic cytotoxic T lymphocytes, while the thymic repertoire is determined solely be the thymic MHC determinants.

Significance to Biomedical Research and the Program of the Institute: The above project will allow a better insight into the mechanisms responsible for the regulation of T lymphocyte responses to foreign antigens and thereby yield ways to manipulate immune disorders in humans which are a consequence of regulatory failure.

Proposed Course of Project: The project will next investigate whether also in other experimental modesl, besides a thymus-determined repertoire, an extra-thymically determined T cell self-MHC specific repertoire can be detected.

Publications:

Kruisbeek, A. M., Hodes, R. J., and Singer, A.: Cytotoxic T lymphocyte responses by chimeric thymocytes: Self-recognition is determined early in T cell development. J. Exp. Med. 183: 13-29, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05095-02 I

PERIOD COVERED

October 1, 1980 to September 30, 198

TITLE OF PROJECT (80 characters or less)

Regulation of Cell-Mediated Immunity by Germ Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	U. Hurtenbach	Visiting Associate	I NCI
Other:	G. M. Shearer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER x

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Autologous mouse testicular cells derived from the seminiferous tubules activate suppressor T cells which inhibit a syngeneic or allogeneic mixed cell reaction in vitro. Generation of cytotoxic T cells in vitro is reduced in the presence of syngeneic germ cells (spermatozoa from the simininferous tubules or epididymal sperm). Spleen cells from mice injected with syngeneic sperm show a nonspecifically suppressed potential to generate cytotoxic T cells in vitro. In contrast, spleen cells from mice injected with syngeneic testicular cells derived from the seminiferous tubules exhibit a reduced response against TNP-modified self antigen; spleen cells from mice inoculated with allogeneic testicular cells from the seminiferous tubules suppress the cytotoxic response against splenic stimulators of the same haplotype.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The objective of this study is to investigate the antigenic, genetic and cellular requirements leading to an autoimmune reaction against male germ cells. For this purpose, murine male germ cells have been chosen as antigen. Due to their morphological isolation, immunological tolerance is not acquired, and thus their cells remain autoantigenic.

Methods Employed: Sequential protease treatment of murine testes released two fractions of cells: a population consisting of interstitial cells; and a second population which is derived from the seminiferous tubules consisting of spermatozoa. Sperm cells were obtained from the epididymis. Spleen cells from normal mice of various strains were sensitized in vitro against autologous spleen cells modified with a trinitrophenyl group or against allogeneic stimulators in presence of autologous germ cells. In another protocol spleen cells from mice previously injected with germ cells were stimulated in vitro against modified syngeneic or allogeneic spleen cells. Lymphocyte reactivity was assayed by measuring the proliferative response by ^{3}H thymidine incorporation and by determining cytotoxic activity on ^{51}Cr-labelled PHA blast cells.

Major Findings: The two testicular cell populations showed different effects by lymphocyte proliferation in vitro: lymphocyte reactivity was suppressed against the autologous germ cell population itself as well as against autologous or allogeneic interstitial cells or against allogeneic spleen cells when germ cells were present during the sensitization phase. In contrast, the testicular cells consisting of an enriched population of interstitial cells stimulated lymphocyte proliferation. The reactive lymphocytes were T cells; suppression could be abrogated by treatment of the responder cells with anti-Ly 2.2 sera, plus complement. Lymphocyte proliferation was significantly reduced by anti Thy 1.2 plus complement treatment. Similar suppressive effects of autologous germ cells have been observed on the generation of cytotoxic T lymphocytes in vitro. In the presence of spermatozoa from the seminiferous tubules the reactivity against modified self or alloantigen was reduced, whereas interstitial testicular cells had no significant effect. No difference has been found in responder spleen cells between male or female mice.

Spleen cells from young adult male mice injected i.v. with syngeneic sperm and then followed by sensitization in vitro against splenic stimulators exhibited strongly reduced potential to generate cytotoxic T lymphocytes against modified self and to a lesser extent, against alloantigens. Co-cultivation of these spleen cells with normal responder cells showed that the lack of cytotoxic reactivity was due to a suppressor mechanism. Suppression was more pronounced with increasing age of the sperm donor mice. The affected recipients exhibited symptoms of graft vs. host disease of various degrees. So far, haplotype restriction has not been found. Injection of germ cell from the seminiferous tubules (which contain immature spermatozoa of various differentiation stages) followed by in vitro sensitization against splenic stimulation also showed

reduced cytotoxic potential. However, in contrast to the unspecific suppression induced by syngeneic sperm, the testicular cell induced suppression was specific. Thus, injection of syngeneic testicular cells inhibited the generation of a CTL response against modified-self only, whereas injection of allogeneic testicular cells exclusively inhibited the response against the stimulators of the inoculated haplotype, but not against a third-party stimulator.

Significance to Biomedical Research and the Program of the Institute: Antigens have been shown to be expressed on cells of the male germ line as well as on tumors which derive from embryonic cells. Both cell types induced immunosuppression. Therefore, this project may be of medical relevance, since it may help to understand the immune status of individuals if such antigens come into contact with the immune system; e.g. after vasectomy or during development of neoplastic cells.

Proposed Course of Project: Experiments will be performed to investigate the mechanism leading to the germ cell-induced suppression of the CTL response. The antigenic determinants on the germ cells responsible for the induction will be investigated using monoclonal reagents. It will be tested (a) whether antibodies directed against specific surface structures bind to germ cells and (b) whether induction of suppression can be prevented by preinjection of the specifc monoclonal reagents. In addition, variation of antigen expression will be studied, since induction of suppression seemed to be related to the age of the germ cell donors. The target cell of the germ cell within the lymphoid cell population will be determined using physical or serological separation methods and consecutively their suppressive ability on normal spleen cells in co-cultivation experiments will be tested. Recombinant mouse strains will be used to determine whether there is genetic restriction at the level of the germ cells for induction of suppression and/or at the level of the interaction of the suppressor cells with the target lymphoid cell.

Due to the fact that another more interesting project is currently being pursued by the P. I. (See Z01 CB 05088) considerable efforts on this project have not been made during the past year. However, a manuscript is in preparation and it is anticipated that this project shall be completed in 1981-82.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05096-02 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Identification and Function of Intracellular Calcium-Containing Organelles

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. P. Henkart	Expert	I NCI
Others:	L. E. Waters	Biol. Lab. Tech.	I NCI
	C. E. Fiore	Physical Scientist	BEIB, DRS

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 21205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to determine the distribution of calcium in cellular organelles and the effects of physiologic stimuli on the distribution of calcium and the morphology of calcium-containing organelles. It is particularly concerned with the question whether the endoplasmic reticulum (ER) is a calcium uptake system and whether calcium can be released form the ER in response to surface membrane stimuli such as specific ligand-receptor interactions. Tissue is prepared by rapid freezing or by modified fixation procedures designed to retain Ca in situ. Ca is identified in sections by electron-probe x-ray microanalysis. Early stages of this study have been devoted to refinement and testing of the methods.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The general objective of this project is to study the distribution of calcium within cells and to determine how its distribution is affected by physiologic stimuli. A working hypothesis underlying a part of this study is that the endoplasmic reticulum (ER) of all cells can function as a calcium sequestering system similar to the sarcoplasmic reticulum of muscle. An implication of this hypothesis is that there may also be a mechanism by which calcium can be released from the ER in response to surface membrane stimuli, providing for a trans-membrane signal in many cell types analogous to excitation-contraction coupling in muscle.

Methods employed: Basic morphologic studies are done using standard techniques of electron microscopy. Identification of intracellular calcium-containing organelles require in addition: 1) Methods of tissue preparation that retain calcium in situ. Two approaches to this have been used: (a) In previous studies (in collaboration with Dr. T. S. Reese of NINCDS) cells were rapidly frozen and substituted with acetone in the presence of osmium. (b) Modified fixation procedures have also been used. These include fixation in aldehyde in the presence of oxalate followed by post-fixation in osmium in acetone. The results obtained by method (b) were compared with results obtained by method (a) using the squid giant axon and mouse skeletal muscle as test tissues. (2) Identification of clacium in the organelles of cells [prepared as in (1)]. This is done by electron-probe x-ray microanalysis using the analytic electron microscope facility being developed by BEIB in DRS.

Major Findings: In previous studies related to this problem I have shown that the endoplasmic reticulum of neurons is a calcium-sequestering compartment. In macrophages areas of both smooth and rough endoplasmic reticulum also contain calcium. The ER of macrophages forms morphologically specialized appositions (subsurface cisterns) with the surface membrane (or the membrane of newly internalized pinosomes) similar to the junctions between the surface membrane and sarcoplasmic reticulum of muscle at "triads". Other organelles also contain calcium. One general class includes organelles whose membranes circulate through the surface membrane via endocytosis and exocytosis. This class includes pinosomes and phagosomes, lysosomal structures and secretory granules. Some cisterns and vesicles in the vicinity of the Golgi apparatus also contain dense deposits, but these are vey small structures and it has not yet been possible to identify them in the images thus far obtained under conditions for x-ray analysis. Calcium has also been identified in the periphery of lipid droplets and in the space between the inner and outer mitochondrial membranes, but rarely is found in mitochondrial matrices.

Significance to Biomedical Research and the Program of the Institute: The role of calcium as a transmembrane signal or second messenger and its importance as a regulator of many intracellular functions is becoming increasingly apparent. Examples of calcium-regulated functions include: motility based on actin-myosin systems, secretion by exocytosis, control of membrane permeability to other ions, processes dependent upon polymerization of microtubules, the activity of many enzymes, and probably control of cell proliferation and differentiation. How calcium is distributed within cells and how its

distribution is affected by physiologic stimuli are, thus, questions of fundamental importance for the understanding of normal cell function. Although many studies have suggested that calcium may be important in control of cell growth, no unified hypotheses have emerged about mechanisms. Identification of calcium in its morphologic context may help to clarify some of the intricacies of cellular control of calcium and, thus, lay the foundation for future studies directed at the role of calcium in the cell biology of cancer.

Proposed Course of Project: Plans for this project still involve refinements of the techniques and determination of their limitations. Because of the failure of the supplier to deliver on schedule the apparatus required for rapid freezing, the development of this technique has been delayed. The analytic EM facility is also still under development. Until now the imaging capabilities of the BEIB microscope under conditions for x-ray analysis have been inadequate for identification of many of the organelles of interest. Further improvement of computer image processing is expected to provide a substantial improvement in imaging unstained material. When these techniques and facilities are available we expect to be able to continue with plans outlined in the annual report for 1979-80.

Publications

Henkart, M.: Identification and function of intracellular calcium stores in neurons. Introduction to Symposium. Fed. Proc. 39:2776-2777, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05098-01 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Non-H-2-linked genetic control of cell-mediated cytotoxic responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
	P. K. Arora	Visiting Fellow	I NCI
	H. Fujiwara	Visiting Associate	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Spleen cells from mice of different inbred strains sharing the same H-2 haplotype but differing in their non-H-2 genetic background were compared for their ability to generate cytotoxic T lymphocyte (CTL) responses to syngeneic cells modified with the trinitrophenyl hapten (TNP-self). In both primary and secondary responses, high and low CTL strains were observed (i.e. non-H-2-linked Ir gene control). Among H-2^d strains the BALB/c was a high responder strain, whereas DBA/2 and B10.D2 were low responder strains. Among H-2^K mice, C3H, AKR/J and B10.BR were the respective high , intermediate, and low responders. Of the H-2^b strains studied C57BL/6 were high, whereas C3H.SW and C57BL/10 were low responder strains to TNP-self. By using different combinations of responding, stimulating and target cells, it was found that these non-H-2-linked differences were not attributable to stimulating or target cells. These studies raise some interesting issues concerning the role of non-major histiocompatibility complex (MHC) genes in regulating CTL responses to foreign antigens recognized in association with self MHC gene products.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: It has been previously demonstrated that H-2 linked genes which map to the K and D regions regulate the CTL responses to TNP-self. The objective of this project was to determine whether non-H-2 linked genes also have an effect on regulating the CTL response to TNP-self. If so, it is also the objective of this project to determine whether the thymus (which is known to influence the expression of H-2 linked genetic control of H-2 restricted CTL response) will affect non-H-2 linked Ir gene control of CTL.

Methods Employed: For in vitro generation of CTL, mouse spleen cells were sensitized in vitro to syngeneic cells conjugated with trinitrobenzene sulfonic acid (TNP-self), and the effector cells generated were assayed on the appropriate ^{51}Cr-labelled target cells. Mice whose spleen cells were to be used for in vitro sensitization to TNP-self were primed in vivo by skin painting with trinitrochlorobenzene.

H-2 matched allogeneic chimeras were prepared by irradiating recipient mice with 850R and by transferring bone marrow cells from donors, involving high responder recipients grafted with low responder stem cells and vise versa. Two-to-four months after cell transfer, the spleens of the chimeras will be tested in vitro for high and low response patterns to TNP-self.

Major Findings: Both in primary in vitro and in secondary in vitro (following in vivo priming) CTL studies the following non-H-2 linked high and low genetic control patterns were observed: among H-2^d strains--Balb/C, high responder; DBA/2 and B10.D2, low responders; among H-2^K strains--C3H, high responder; AKR/J, intermediate responder; B10.BR, low responder; among H-2^b strains--C57BL/6, high responder; C34H.SW and C57BL/10, low responders. These differences were observed to be more pronounced in the secondary than in the primary response, and radioresistant helper T cells were demonstrated to be involved in at least part of the differences among high and low responder strains. By varying the strains used for providing responding, stimulating and target cells, it was found that the low responder patterns could not be accounted for by stimulating or target cell defects. Therefore, these difference in high and low responder strains are likely to reside among the helper, CTL precursor, and/or accessory cells provided by the responding cell pool.

Significance to Biomedical Research and the Program of the Institute: Over the last 15 years considerable emphasis has been placed on the importance of MHC linked Ir genes in the control immune responsiveness, and such regulation has been demonstrated both in experimental animal models and man. Based on the dramatic effects that the thymus has on both MHC restriction and on phenotypic expression of Ir genes, it has been postulated that Ir gene patterns of responsiveness are reflections of MHC restriction. The potential significance of the present project is that it demonstrates that Ir genes which are not linked to the murine MHC also have a dramatic effect on T cell

immune responses--even those which are MHC restricted. Such studies underscore the fact that in considering the genetic regulation of immune potential, heredity effects other than just those linked to the MHC must also be considered.

Proposed Course of Project: Among the strains thus far investigated, F_1 hybrids and backcross mice will be tested to establish whether high or low responsiveness is dominant and to obtain an estimate of the number of genes involved. Since these high and low responders are H-2 compatible, allogeneic irradiation chimeras are being prepared to determine whether high responsiveness is a characteristic of the host environment or of the donor stem cells. It may also be important to do thymic grafts in athymic nude mice to determine the role of the thymus in such Ir gene control. Congeneic mice differing at other known non-MHC markers will be compared to determine if there is linkage to other loci (e.g., allotype). CTL responses to other haptens plus self as well as to alloantigens will be investigated to determine how broad non-H-2-linked regulation of CTL responses are. Responding cell populations will be fractionated to attempt to define a particular cell population(s) which may express the genetic defect in low responder strains.

Publications

Fujiwara, H., and Shearer, G. M.: Non-H-2-associated genetic regulation of cytotoxic responses to hapten-modified syngeneic cells: Effect on the magnitude of secondary response and helper T cell generation after in vivo priming. Eur. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-05099-01 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunogenetic effects of murine cytomegalovirus on induced and natural immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	G. M. Shearer	Senior Investigator	I NCI
OTHERS:	J. Chalmer	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mice injected with sublethal doses murine cytomegalovirus (MCMV) exhibit rapid and dramatic changes in their ability to generate in vitro cytotoxic T lymphocyte responses to hapten-self and to alloantigens. Within three days after intraperitoneal injection of (MCMV), the CTL responses to hapten-self and alloantigens are abrogated or severely reduced. This is followed by rapid recovery to a normal level of CTL potential, and then to a hightened state of immune potential detected by the hapten-self CTL only. The injection of F_1 hybrid mice with either MCMV or parental spleen cells resulted in rapid and severe immunosuppression. Inoculation of either the virus or parental cells were selected so that they would be below the threshold for severe immunosuppression. However, when these two inocula were combined, severe immunosuppression was observed. These studies permit the investigation of the immunosuppression of MCMV infection and the possibility consequences of CMV infection coupled with a graft-versus-host reaction (GVHR).

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The purpose of this project is to investigate the immunological and genetic effects of MCMV infection and of MCMV infection plus a GVHR on (a) acquired T cell immunity to hapten-self antigens and alloantigens; and (b) on natural resistance to MCMV infection and the GVHR. Since certain mouse strains are relatively resistant to MCMV and to parental T cell-induced GVHR, it will also be the purpose of this study to investigate the role of H-2-linked and non-H-2-linked genetic effects of resistance and susceptibility to MCMV, to GVHR and to a combination of MCMV and GVHR.

Methods Employed: Sublethal doses of MCMV (prepared from salivary glands of infected mice) were injected intraperitoneally into various inbred and F_1 hybrid mouse strains. Also F_1 mice were injected intraveneously with known concentrations of parental spleen cells, and F_1 mice were also injected with MCMV plus parental cells. The T cell immune potentials of injected and control mice were tested by in vitro sensitization to hapten-self and alloantigens, and the CTL activity was determined 5 days later using the ^{51}Cr-release assay.

Major Findings: The injection of sublethal doses of MCMV resulted in rapid suppression of CTL potential to both hapten-self and allogeneic antigens (within 3 days). This was followed by recovery (by around 7 days), and augmented CTL activity as detected by the hapten-self and not by the allogeneic CTL systems (days 9-13). The injection of F_1 mice with doses of MCMV plus parental spleen cells each of which alone did not drastically reduce CTL potential, resulted in synergistic effect which abrogated CTL potential.

Significance to Biomedical Research and the Program of the Institute: Cytomegalovirus infection is one of the major problems currently facing human bone marrow transplantation, and may become critical in patients undergoing a chronic GVHR. An understanding of the genetic and mechanistic paramaters involved in resistance and susceptibility to CMV in the murine model, the immunosuppression associated with CMV infection, and the possible synergistic effects of CMV infection and chronic GVH should be valuable for both basic and clinical purposes.

Proposed Course of Project: A number of inbred, recombinant and F_1 hybrid mouse strains will be studied for their ability to be resistant or susceptible to immunosuppression rsulting from MCMV infection. We shall also investigate the genetic and mechanistic aspects associated with the synergistic effects of MCMV and the GVHR on immunosuppression.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05100-01 I

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
The Role of HLA Genes in Human Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Shaw	Senior Investigator	I NCI
Other:	R. Hall	Clinical Associate	Derm NCI
	S. Katz	Chief, Dermatology Branch.	Derm NCI
	H. McFarland	Asst. Chief, Neuroimmunology Br.	NI NINCDS

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have recently defined a new HLA gene which maps centromeric to the other known genes of the HLA complex. Studies have now been initiated to study the distribution of SB antigens in two different disease populations in order to further understand the role of HLA genes in determining disease susceptibility--multiple sclerosis and dermatitis herpetiformis. In the 38 unrelated MS patients studied, there was no significant deviation in the frequency of SB antigens, relative to 200 normal control donors. However, among the 41 DH patients studied, there was a significant deviation from the normal donors, particularly with respect to an elevation of the frequency of the SB1 antigen and a decrease in the frequency of the SB2 antigen. These data cannot be explained by the known strong association between DH and DR3; rather they indicate that there is an interaction between the HLA-SB and the HLA-DR phenotype in determining the risk for dermatitis herpetiformis.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Previous studies from many laboratories have demonstrated that there are associations between many specific diseases and particular HLA antigens. For virtually all of these associations it is not known: 1) exactly what gene product is involved in the disease pathogenesis (i.e., whether the HLA gene product identified is involved in the disease or some unknown allele with which it is in linkage disequilibrium); and 2) what the mechanism is for the association. The extraordinary number of HLA associated diseases and the overall importance of the HLA region in immune responses suggest that an understanding of these associations may be of rather general relevance.

As we develop new markers of the HLA region (project Z01-CB-05101 I), we expect they will be useful in helping us to map more precisely the gene products involved in these disease associations.

Methods Employed: Patients are selected by diagnostic criteria relevant to the particular disease. Patients peripheral blood lymphocytes are "typed" for SB antigen expression by the primed lymphocyte typing techniques outlined in project Z01-CB-05101 I; they are also serotyped for other HLA antigens under contract N01-CB-04337.

Major Findings: 38 unrelated patients with multiple sclerosis were studied. The distribution of SB antigens in this population did not differ from that in 200 normals. Thus, despite a modest increase in frequency of DR2 (and B7) in MS patients there is no marked alteration of SB antigens. This suggests that the HLA gene product involved is more closely associated with DR than SB.

In contrast, in studies of dermatitis herpetiformis, a striking alteration of SB antigen distribution was observed. The results of typing on the first 12 patients were analyzed retrospectively, hypotheses were formulated, and tested on the next 29. In both groups there was a striking increase in the frequency of SB1 (42% vs. 11% in normals) and decrease in SB2 (12% vs. 31%). These alterations could not be explained by the known strong association of DR3 and DH. Instead they suggested that among individuals with DR3, the risk of DH was increased about 2-3 fold by having the SB1 antigen but decreased about 5 fold by having the SB2 antigen. Thus, there is an interaction between the SB and DR phenotypes in determining the risk for DH.

Significance to Biomedical Research and the Program of the Institute: Many diseases are known to be HLA associated, including certain malignancies. Understanding of the role of HLA genes in the pathogenesis of these diseases might reasonably be expected to help in therapy and prevention of these diseases.

Proposed Course of Project: The information on DH is consistent with two genetic models. First, it is possible that there are two independent HLA-linked "disease control genes": DR3 increases the risk of DH (or an allele of another gene in strong positive linkage disequilibrium); SB2 decreases the risk for DH (or an allele of another gene in strong positive linkage disequilibrium with SB2). Second, it is possible that there is a single "disease control gene"; this disease gene does not occur randomly on HLA haplotypes, but predominantly on DR3-positive, SB2-negative haplotypes. Differentiation of these two hypotheses will be possible with family studies of DH patients.

With the SB marker system it will be informative to examine a number of other diseases which are known to be associated with DR3, such as juvenile onset diabetes mellitus and myasthenia gravis. This should help resolve whether all of these diseases have in common a gene which predisposes to autoimmunity, or whether they have different disease genes, each of which is in positive linkage disequilibrium with DR3.

Publications

Shaw, S., and Shearer, G. M.: Cytotoxic T cell interactions with antigen: potential relevance for drug-related systemic lupus erythematosus. Arthritis Rheum., 1981, in press.

Kaslow, R. A. and Shaw, S.: The role of HLA in infection: A review and perspective. Am. J. Epidemiol., 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05101-01 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Definition of Gene Products of the Human Major Histocompatibility Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Shaw	Senior Investigator	I NCI
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	G. Pawalec/P. Wernet	Tubingen, Germany	
	A. Termijtelen/J.J. van Rood	Leiden, Netherlands	
	C. Mawas	Marseille, France	
	P. Kavathas	Stanford U., Stanford CA	
	R. Duquesnoy	Milwaukee, WI	

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8 (NIH)	0.6 (NIH)	0.2 (NIH)

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using two different cell-mediated typing assays (secondary lymphocyte proliferative responses and secondary cell-mediated cytotoxicity) we have defined five new HLA-linked antigens designated "SB antigens" (secondary B cell). A reliable system of primed lymphocyte typing for these antigens has been perfected using frozen reagents, and a statistical algorithm for scoring the results is used. Genetic studies suggest that the SB antigens are encoded by a single HLA-linked gene. These antigens have striking similarities to the DR antigens with respect to genetics, tissue distribution and function. However, studies of recombinant families and of mutant cell lines demonstrate that the SB antigens are encoded by a gene distinct from DR, which maps between HLA-DR and GLO. The discovery of this new gene in our laboratory has prompted a large number of collaborations, both to utilize the expertise of other laboratories, and to help others learn to define the SB gene products.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Genes of the HLA region are important in tissue transplantation, immune regulation and disease association. Consequently, it is crucial to define the gene products of this region and to determine the function of these gene products. There has been a worldwide effort to do so, primarily by serologic techniques. We believe that T cells may be the most sensitive probe for defining intricacies of the HLA region, since T cells seem to be uniquely committed to recognizing gene products of this region. Therefore we expect refined approaches to cellular typing to allow definition of new HLA gene products. Once appropriate tools have been found to define these antigens, we will investigate the genetics of these markers, the tissue distribution of the markers, and function of the markers in cellular immune responses.

Methods Employed: Human PBL are obtained from donors by phlebotomy or batch leukapheresis; mononuclear cells are separated by density separation, and cryopreserved. Analysis of the serologically defined HLA markers on the donors cells is performed by microcytotoxicity testing under contract N01-CB-04337. Cells from carefully selected donors are sensitized in vitro in one way mixed lymphocyte culture, and generally the primed cells are restimulated after 10 days with the same stimulator to enhance weak responses. Primed cells are frozen in large batches, and thawed as necessary to provide a standard "reagent". Proliferation of these cells in response to stimulator cells is measured by ^{3}H-thymidine incorporation. Cytotoxic activity is analyzed by short term ^{51}Cr release assays using as targets T lymphoblasts or lymphoblastoid B cell lines. The assignment of specificities to a donor's cells is made on the basis of a statistical technique called centroid cluster analysis, which has been improved and adapted for this application.

Major Findings: Five new histocompatibility antigens, designated secondary B cell (SB) antigens have been identified by secondary allogeneic proliferative and cytotoxic responses. The reagents used to define the SB antigens are lymphocytes primed between donors matched for all previously known HLA antigens. To standardize typing for these antigens, cryopreserved primed lymphocytes are now used as standard reagents, and a technique of cluster analysis has been modified to score typing results objectively. Two primed reagents are used to define each SB antigen; although derived from independent responder-stimulator combinations, the concordance between reagents is good. Using this carefully controlled approach to typing, we have studied the genetics of these SB antigens in populations and families. The SB antigen distribution in a population of 215 normal donors is consistent with Hardy-Weinberg equilibrium of alleles of a single locus. Estimated gene frequencies ranged between 3% (SB5) and 36% (SB4) with approximately 31% blanks. Analysis of association between the SB and HLA-A,B,DR antigens in 200 normal donors revealed that associations were generally weak with a few exceptions, in particular the A1,B8,DR3,SB1 haplotype and also the B7,DR2,SB5 haplotype. The segregation of the SB antigens has been studied in 25 families. In all cases the inheritance is consistent with autosomal dominant inheritance of alleles of a single locus. The SB locus is tightly linked to the other HLA genes, but is separable by recombination. The results of 7 HLA-recombinant families indicates that the SB locus maps between HLA-DR and GLO.

The genetics of the SB locus have also been studied in gamma ray induced HLA-mutant cell lines. The conclusion that HLA-DR and SB are independent genes has been confirmed by the observation that some of these mutant lines have lost HLA-DR, but not the product of the cis-linked SB gene.

The tissue distribution of the SB antigens has been examined by studying different cell types as targets for (and inhibitors of) cell mediated lysis. These studies indicate that the SB antigens are selectively expressed on B cells and macrophages, but weakly, if at all, on T cells.

The structure of the SB antigens cannot be definitely determined until the molecule can be isolated. At present, no alloantisera have been identified which recognize the molecule. However, the best available evidence suggests that SB antigens have a molecular structure similar to HLA-DR--a two chain glycoprotein with 33,000 and 29,000 mw chains. This is inferred from the finding that a monoclonal antibody which brings down only material of that description is able to inhibit some SB-specific CML.

The SB antigens have been studied for their function in allogeneic cellular immune responses. They induce a small primary proliferative and cytotoxic response, but this response is augmented in secondary responses. Of particular interest is the finding that the proliferative and cytotoxic responses to the SB antigens are not "restricted" by the other known HLA antigens; this suggests the SB antigens are "major" histocompatibility antigens not only because they are encoded by a gene in that region, but because they function in cellular immune responses independently of the other HLA antigens.

Similar studies are just beginning in two other areas which may reveal new systems of HLA antigens. One of these is a system of antigens which is recognized by cytotoxic T cells on cells which express the HLA-Bw44 antigen. At present two antigens can be defined. Indirect evidence suggests that the gene encoding these antigens (or closely associated antigens) may control the expression of the Bw44 molecule on platelets (itself an unexplained phenomenon) and may regulate influenza-specific immune responses to HLA-Bw44.

Significance to Biomedical Research and the Program of the Institute: Because genes of the HLA region are crucial in controlling immune responses, transplantation, and increasing the risk of a large variety of diseases, therapeutic intervention related to these phenomenon may depend on further understanding of the genes in this region. The SB gene defined already in this project promises to be a very informative one. Since it maps quite a distance from the other known HLA markers, it will provide an important new marker for population studies. Furthermore, the SB gene product itself may be important in immune regulation and disease. Its similarities to HLA-DR and the murine Ia antigens suggest that it may be involved as an Ir gene (controlling immune responses). Furthermore, initial studies (project Z01-CB-05100 I) suggest that it may be a useful new genetic marker for the disease dermatitis herpetiformis.

Proposed Course of Project: We plan to pursue functional studies of the SB antigens with respect to their role in cellular immune responses to foreign antigens and their possible function as Ir genes (probably under project Z01-CB-05067 I). Furthermore, we expect to be involved in structural studies of the SB molecule by identifying antibodies (monoclonal or conventional) which interact with the SB molecule and collaborate with a laboratory able to do structural studies of the relevant molecule. We will continue studies of the relevance of this new marker system in disease (project Z01-CB-05100-01 I).

This work has attracted considerable attention from the world community of scientists interested in HLA. Of necessity, we will be the world reference laboratory for defining the SB antigens, until other laboratories are prepared to assume this function.

We will also be pursuing this approach in definition of other segregant series of antigens such as the Bw44 related antigens alluded to above.

Publications

Shaw, S., Pollack, M. S., Payne, S. M. and Johnson, A. H.: HLA-linked B cell alloantigens of a new segregant series: population and family studies. Hum. Immunol. 1: 175-177, 1980.

Shaw, S., Johnson, A. H., and Shearer, G. M.: Evidence for a new segregant series of B cell alloantigens which are encoded in the HLA-D region and stimulate secondary allogeneic proliferative and cytotoxic responses. J. Exp. Med. 152: 565-580, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05102-01 I

PERIOD COVERED

October 1, 1980-September 30, 1981

TITLE OF PROJECT (80 characters or less)

Morphologic Studies of Cellular Interactions in the Immune System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. P. Henkart	Expert	I NCI
Others:	P. A. Henkart	Chemist	I NCI
	P. J. Millard	Biologist	I NCI
	T. T. Timonan	Visiting Associate	LID NCI
	J. R. Ortaldo	Biologist	LID NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.9	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒(b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Interactions of positively identified human natural killer (NK) cells known to be killing appropriate target cells have been studied in serial thin sections with the electron microscope. NK cells have a characteristic type of granule containing bundles of tubules in crystalline arrays. The tubule bundles resemble structures formed by microtubule protein (tubulin) under some conditions. Within an hour of binding to a target cell several types of granule within the killer cell, including tubule-containing granules appear to fuse and the heterogeneous contents of the fused granules are secreted by exocytosis into the space between the killer and target cell. The secreted material is mostly in the form of membrane-like structures some of which have ring-shaped profiles on their surfaces. The morphology suggests that tubulin associated with membrane vesicles is released from the killer cell. The secreted products cling to and possibly fuse with the target cell membrane.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: (1) To study the ultrastructure of interactions between identified cell types involved in various aspects of immune responses and (2) To correlate morphologic observations with functional studies. The first system being approached in this way is the interaction of human natural killer cells with appropriate target cells.

Methods employed: Basic morphologic studies are done using standard techniques of electron microscopy. For studies of particular cellular interactions study of serial thin sections is necessary. Special stains and extracellular tracers are used as required. Immunocytochemistry using fluroescent labelled antibodies for light microscopy and ferritin-labelled antibodies at the EM level are used for identification of cellular constituents.

Cells are obtained from peripheral blood of normal adult human donors. Fractions of lymphocytes from discontinuous Percol gradients are prepared by published techniques (Timonen & Saskela, J. Immunol. Methods 36: 285, 1980). Killer-target conjugates are made by centrifuging together and rediluting the the natural killer cell enriched lymphocyte fraction with NK susceptible targets, the human leukemia cell line, K-562. To ensure that all lymphocytes forming conjugates are active killers, the lymphocytes are treated for 2 hours before conjugate formation with human interferon. Under these conditions virtually all lymphocytes that bind target cells kill them within 4 hours.

Major findings: All positively identified NK cells as well as a high proportion of the cells from the percol gradient enriched for NK activity contain a characteristic type of cytoplasmic organelle which is a membrane enclosed bundle of tubules some of which may be in crystalline arrays. (These tubule bundles had previously been observed by us and shown to be a characteristic feature of human peripheral blood lymphocytes bearing receptors for the Fc portion of the IgG molecule as defined by their adherence to antigen-antibody complexes.) The detailed structure of these organelles and their sensitivity to vinblastine suggested that they contained microtubule protein. NK cells also contain a variety of other granules distinguished on the basis of their morphologies.

In NK cells that have formed conjugates with appropriate targets the contents of granules becomes more heterogeneous, apparently because of fusion of several granule types. In some cases tubule bundles are seen in the same granules as numerous small vesicular structures similar to those contained in classical multivesicular bodies. Material similar in appearance to the heterogeneous granule contents including fragments of tubule bundles and membrane vesicles are also found in the extracellular space between killer and target cell. The membranous extracellular material sometimes bears superimposed ring-like profiles similar in diameter to microtubule cross-sections. The simplest interpretation of the morphologic observations is that the mixed contents of the granules

are secreted by exocytosis. The images seen in the EM suggest further that the membranous material released from the killer cell may fuse with the target cell.

Significance to Biomedical Research and the Program of the Institute: Ultrastructural studies of cellular interactions in the immune system promise to contribute much to the understanding of mechanisms of immune functions. Until recently, however, this has not been profitable approach because of the heterogeneity of cell types involved in immune responses. With the recent development of techniques for isolation, characterization, and, in some cases, cloning of lymphocyte populations with specific functions it has become feasible to study cellular interactions at the ultrastructural level.

Attack of tumor cells by cytotoxic lymphocytes is probably important in normal defense against neoplasms. Understanding of the mechanisms by which cytotoxic effector cells kill their targets is fundamental to the potentially useful ability to manipulate cytoxic lymphocyte function for prevention or therapy of neoplasms.

Proposed Course of Project: Immunocytochemistry with anti-tubulin antibody will be used to determine whether the tuule bundles actually contain tubulin and whether tubulin is secreted and transferred to target cells. We intend to compare the interactions of NK cells and their targets with the interactions of antibody-dependent cytotoxic effectors and cytotoxic immune T cells with their respective targets to determine whether similar mechanisms are involved.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05006-16 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Lymphocyte Cell Surface Antigens, Normal and Neoplastic

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Mann	Senior Investigator	I NCI
Other:	C. Murray	Guest Worker	I NCI

COOPERATING UNITS (if any) William Blattner and Mark Green, Epidemiology Branch, NCI S. Broder and A. Muchmore, Metabolism Branch, NCI, B. Haynes, Laboratory of Immunology, NIAID, and David Poplack, Pediatric Oncology Branch, NCI

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Biologic, functional and chemical characterization of cell surface structures of normal lymphoid and malignant cells are under investigation. Antigen (HLA) controlled by the major histocompatibility complex (MHC) have been assessed in diseased populations and at risk families. Results indicate that several interacting genes and gene orientation predispose to disease states and immune response. Immunoprecipitation analysis of antigens controlled by the MHC DR gene region suggest at least 3 gene products. Antisera detecting some of these antigens inhibit antigen stimulation and Ig synthesis when these sera are reacted with monocytes and acute lymphocyte leukemia cells. This inhibition has been determined to be due to suppressor T cell activation. 3 monoclonal antibodies 3A1, 4F2 and 5E9 have been characterized for antigen specificity molecular weight of antigen detected and normal cellular distribution of antigens. These monoclonal antibodies have been used to determine phenotypes of malignant cells of lymphoid origin. Cells from patients with Sezary syndrome are $3A1^{-}$ $4F2^{+}$, a phenotype not found on normal cells. Acute lymphocytic and myeloid leukemia cells exhibit a wide variation in antigen expression suggesting dedifferentiation of cells undergoing malignant transformation.

Project Description

Objectives: The objectives of these studies are as follows: a) To identify genes in the major histocompatibility complex which have an association with disease states; b) to identify potential immune response genes in man; c) to assess the biologic function of different cell surface structures in relationship to immune function of lymphocytes; d) to isolate and purify gene products of the major histocompatibility complex for structural analysis; and e) to identify lymphocyte cell surface markers using monoclonal antibodies which react with functional subsets of lymphocytes.

Methods Employed: Gene products of the major histocompatibility complex are identified by dye exclusion cytotoxicity tests. In order to assess the presence of these antigens, subpopulations of cells were isolated as follows: monocytes are recovered after adherence to a glass surface using an EDTA reagent. The non-adherent cells are then plated on plastic surfaces previously coated with an antiserum to the F(ab) component of Ig. This technique isolates the immunoglobulin bearing B lymphocyte. These cells are removed by competition with human Ig. The remaining cell population is considered the T cell population and is then used for cell surface antigen identification or recombined with the other subpopulations of lymphocytes isolated by the above procedures for biologic study. The frequency of HLA alloantigens in a disease population are compared with frequencies in a normal population and/or related disease population by Chi square analysis. Antigen associations were determined by comparing frequencies of antigens occurring together in the disease population and compared with the association of these antigens in a normal population. Related disease groups were compared in a comparable fashion. In family studies haplotype association with the disease was determined by lode score analysis.

The generation of suppressor cell function using anti-B cell sera and monocytes was performed as follows. Isolated monocytes were exposed to antigen (SKSD, Candida tetanus toxoid) washed and then reacted with the antisera. In other studies, monocytes were treated separately and then incubated with autologous or allogeneic T lymphocytes. Tritiated thymidine incorporation was measured after 5 days. To measure the suppression of Ig synthesis, hetero anti-B cell serum was incubated with peripheral blood lymphocytes and the cultures stimulated with the mitogen pokeweed. Levels of IgG, IgA and IgM in supernatants from these cultures were measured by a radioimmunoassay.

The interaction of the various cell populations in allogeneic mixed lymphocyte reaction employs the standard techniques of lymphocyte culture using tritiated thymidine incorporation as a measure of response. Cell surface antigens have also been investigated employing flow microfluorometry using the fluorescence activated cell sorter. Lymphocytes or subpopulations thereof were isolated as described above for analysis. Monoclonal antisera products of genes of the major histocompatibility complex are incubated with the cell populations, the cells washed and a fluorescinated goat anti-mouse Ig antibody used to identify the reaction of the antibody to the cell surface.

Monoclonal antisera were also directly labeled with fluorescien and incubated with lymphocytes or subpopulations thereof. After extensive washing, the cells are analysed with the fluorescence activated cell sorter.

Studies of the molecular characteristics of human B cell alloantigens were performed by labelling cell membrane components with tritiated amino acids. The cell membrane components were solubilized with non-ionic detergents and purified by gel filtration chromatography and Conavalin A lectin column purification. After elution of the adhered antigen with alpha-methylmannose appropriate anti-DRw (or anti-human Ia antisera) as well as control sera were incubated with the isolated material and the antigen-antibody complexes precipitated with the Cowan strain Staph. aureus organism. The antigen antibody complexes were dissociated with sodium dodecyl sulfate in the presence of the reducing substance mercaptoethanol. These dissociated materials were electrophoresed on polyacrylamide gels containing sodium dodecyl sulfate. Gel slices were counted in a liquid scintillation counter and the migration of the isolated labelled proteins assessed. In some instances sequential precipitations with the anti-DR sera or anti-Ia sera were performed. In other studies, combinations of antisera were used in precipitation analysis.

Major Findings: HLA-B, C and DRw antigen determinations are being performed in a normal population and in a variety of disease conditions. These studies are carried out in part in this laboratory and supported by an NCI contract N01-CB-53890 to Duke University. The major observations are as follows: analysis of HLA alloantigen profiles in patients with rheumatoid arthritis revealed an association of antigenic determinants controlled by multiple genetic loci in the major histocompatibility complex. HLA-A1, B40, DR4 and MT2 antigens were associated in combinations more frequently than in the normal population. The results suggest the hypothesis that multiple genes within the MHC predispose to the disease state. Studies in several other disease conditions reinforce this hypothesis. Three families with multiple cases of primary and secondary Sjogren's syndrome were studied. The MT1 and MT2 antigens were found in trans haplotype position in only those affected family members with the primary disease. In those individuals with secondary Sjogren's syndrome (rheumatoid arthritis) the MT1 antigen was not present and DR4 and MT2 were found on the trans haplotypes. Patients with psoriatic arthritis were classified as to disease severity. HLA alloantigens found to be increased in frequency in the total diseased population (HLA-A26, Bw38, Cw6, DR7, Ia744) were found to have different associations depending on severity of disease. These results again suggest interaction of multiple MHC genes in disease predisposition. Association of HLA alloantigens were also found in studies of insulin allergic diabetes. The frequency of combinations of HLA-A2, B44, DR7 occurred in 39% of insulin allergic diabetics, 5% in nonallergic diabetics and 32% of normal. The results indicate that gene(s) linked to the genes controlling the expression of the above antigen predispose to allergic reaction in diabetics receiving insulin.

The role of the monocytes in a mixed lymphocyte reaction was studied. The presence of monocytes was demonstrated to be required in either the stimulating or responding cell population in order for stimulation to be observed. In these studies antisera directed to the DR antigens on the monocytes of the responding population were observed to inhibit the MLR. This inhibition was demonstrated to be due to the induction of suppression by these monocytes in the autologous cell population.

These studies have been extended to investigate the mechanism of suppression. Both allo and hetero anti-DR sera inhibit antigen stimulated lymphocyte cultures and pokeweed induced in vitro immunoglobulin synthesis. This inhibition was found to be due to induction of suppressor T cell function based on the following observations. T cells were isolated from lymphocytes cultured with monocytes exposed to anti-DR sera for 24 hours. These T cells effected suppression of Ig synthesis and antigen stimulated lymphocytes. Low doses of irradiation (2000R) in primary cultures reversed the suppression. $F(Ab')_2$ fragments of hetero and allo anti DR sera did not induce suppression. In experiments designed to negate the possibility that anti-DR sera were simple blocking antigen presentation by monocytes, acute lymphocytic leukemia cells were reacted with anti-DR sera and added to autologous lymphocytes. These antisera treated cells were capable of suppression induction. Sera from patients with ALL (active disease and remission) were capable of induction of suppression when reacted with ALL cells.

Monoclonal antibody was produced by the immunization of BALB/c mice with the human HSB-2-lymphoid T cell line. The spleen cells were fused with the P3X63/Ag8 BALB/c myeloma cell line. Antibody from these cell lines have been isolated by ammonium sulfate precipitation and isoelectric focusing. The antibody was labeled with fluorescein or tested in a sandwich technique using goat anti mouse k chain reagent. Six antibodies have been well characterized. 3A1 antibody reacts with 85% of peripheral blood T lymphocytes and precipitates a 40,000 dalton molecular weight molecule from the T cell membrane. The 4F2 antibody reacts with monocytes and thymocytes and with less than 5% of the T cells and precipitates an 80,000 dalton component. The 5E9 antibody detects an antigen on thymocytes and activated T cell population. Three antibodies precipitate molecules of 44,000 and 12,000 daltons and are thought to react with HLA.A, B and C like antigens in that these sera react with all peripheral blood lymphocytes. However cold inhibition studies with these antibodies demonstrate lack of cross blocking of antibody indicating that they are not reacting with the same determinants on the molecule.

These monoclonal antisera were used to study a patients population with Sezary syndrome. Cells from these patients were $3A1^-$, $4F2^+$ Ia^+ thus demonstrating a distinct cell surface antigen phenotype in this malignancy. Cell surface

antigens were also determined on cells from patients with acute lymphocytic (ALL) and myelocytic leukemia (AML). using the above monoclonal antibodies and in addition the OKT3, OKT4, OKT6, OKT8 monoclonal antiserum. The 3A1 antigen was found in relatively high density on T cell ALLs and was not present in appreciable amounts on non T, non B cell ALLs. The patterns of reactivity of the other monoclonal antibodies with ALL cells varied from patient to patient. No constant pattern of combinations of antigens was observed. The reported observation that the patterns of antigen distribution found in normal thymocytes during differentiation could also be found in ALL were not confirmed in this study. The presence of antigens, specific for normal T cells, on non T, non B cell leukemias and on cells from patients with AML supports the hypothesis of cellular differentiation in ALL and AML rather than a malignant clonal expansion of normal cells.

Immunoprecipitation studies with alloantisera detecting B cell alloantigens demonstrate a complex series of molecular components depending on the cell of origin and antisera used. Hetero anti-B cell sera precipitate detergent solubilized components having molecular weights of 34,000, 31,000, 28,000 and 27,000 daltons. Alloantisera detecting different DR MT and MB precipitate combinations consisting of 2 or 3 of the above molecules. The results are consistant with the interpretation that multiple genes control the expression of the B lymphocyte alloantigens.

Significance to Biomedical Research and Program of the Institute: Human B lymphocyte alloantigens appear to be analogous to the B cell alloantigenic system in mice. Genes controlling these antigens in mice appear to be linked to or associated with the ability of the animal to respond to particular antigens. Identification of human alloantigens together with a description of the fine structure of the genetic complex is necessary for the understanding of the relationship of the MHC to disease states. Our studies of the various disease entities described above suggest that multiple genes in the MHC are associated with the disease entity or a manifestation of the disease. Studies in the diabetic population where specific allergic manifestations to insulin or insulin components have been identified, strongly indicate the presence of a specific immune response gene in man.

Monocytes express DR alloantigens and appear important in the initiation of the immune response. The mechanism of communication of the monocyte the T and/or B cells in activation of immune response is unknown. The demonstration that an intact antibody to HLA-DR antigen induces a strong signal for induction of suppressor cells establishes a new concept in immunology. The biologic relevance of this observation relates to a model for control of immune response by a biofeedback mechanism. These observations tend to support the hypothesis that the molecule structures coded for by genes that determine DR antigens serve a role in cell-cell interaction. The observation that leukemia cells bear DR or DR like determinants and that sera from these patients induce immunologic suppression may explain the lack of a normal response to cells undergoing neoplastic change. Since these DR determinants appear to be

different from those found on normal cells, production of antibody to the DR determinants on neoplastic cells may lead to suppression of immune response to the malignant cells. This hypothesis is supported by the observation that a variety of neoplastic cells bear DR like antigens and our observation that sera from patients with leukemia induce suppression when reacted with leukemic cells.

Monoclonal antibodies to determinants expressed differentially on lymphocytes are potential tools in isolation of functional subsets of lymphocytes and the study of interaction of lymphocyte populations in the immune response. Once characterized, these antisera may be used in the identification of alteration of lymphocyte subpopulations in the disease states. One antibody under study has demonstrated reactivity against peripheral blood cells of patients with Sezary syndrome and not reactive with T cells from normal individuals. These sera can thus potentially be used to identify malignant cells for diagnostic or therapeutic procedures.

Studies of molecular components reacting with antisera detecting B cell and monocyte antigens gives insight into the possible role of these cell membrane structures in immune response. Our observation that alloantisera reacting with these antigens precipitates different size molecules depending on the cell source suggest that recombination of several molecules on the cell surface can occur. Such rearrangements may have important influences on cell-cell interactions. Our observations that the DR or Ia like antigens have different associations in disease states than in the normal population suggest that molecular rearrangement of these structures on cell surfaces may predispose to disease condition.

Proposed Course of Project: Studies in the various disease entities mentioned above will be continued. The studies are now and will be directed primarily to families particularly those wherein more than one member has a malignant disease condition. The goal is to determine the potential for cooperative gene effects in the role of the pathogenesis of the disease. Families in which there are multiple cases of malignancies will be HLA typed and studied for in vitro immune response capability. In addition lymphocytes from the family members will be studied for cell surface markers using monoclonal antibodies that are reported to differentiate functional subsets of lymphocytes. Information obtained from this multidirectional approach will be used to attempt to determine the role of the MHC and other genes in immune responsiveness and to attempt to define immune alteration in patients with malignancies. These studies will be performed in collaboration with the family studies section of the Epidemiology Branch, NCI.

Studies will continue in attempts to define specific biologic function of the molecular structures bearing the human DR antigens. These studies will be directed towards understanding the role of these structures in activating or suppressing immune response. These studies will be extended to patients with malignancies to amplify our observations that sera from acute leukemia patients contain substances (non-cytotoxic antibodies?) that induce suppression when reacted with leukemia cells.

Further characterization of the molecular structure of HLA-DR antigens will be undertaken. The specific goals will be to isolate and chemically characterize the molecules that bear the different antigenic determinants.

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SUMMARY REPORT

MACROMOLECULAR BIOLOGY SECTION, IMMUNOLOGY PROGRAM
DCBD, NCI

October 1980 - September 1981

The Macromolecular Biology Section is a small unit in the Immunology Program, reporting directly to the Director, DCBD, NCI. The common interest of the investigators is the elucidation of macromolecular changes on the surface of mammalian cells, and how such changes relate to certain normal (differentiated) cell surface functions pertinent to cell division and to the appearance of cellular tumorigenicity.

Sid Shifrin developed a very productive cooperative study with investigators centered around Dr. L. D. Kohn in the LBP at NIAMDD, on the binding of thyroglobulin to the thyroid receptor in the bovine thyroid plasma membrane. Ingenious chemical modifications of either of the lysyl or of the tyrosine residues of the thyroglobulin, devised by Sid Shifrin, showed that the site of thyroglobulin iodination on the tyrosine residues is the important determinant in the receptor binding process. Another approach, in which the carbohydrate chain of the thyroglobulin was altered by removing enzymatically either sialic acid or galactose, which resulted in the appearance of an altered higher sedimenting form of the thyroglobulin, demonstrated that the recognition of N-acetylglucosamine residues and the extent of iodination in the altered thyroglobulin is also involved in the binding to the specific receptor site. There are large numbers of thyroid tumors which affect the structure of the thyroglobulin through it its biosynthesis, and also affect its biodegradation. Thus the elucidation of the nature of the binding site on the thyroid membrane and the structural features of the thyroglobulin required for binding and degradation has importance to the program of the NCI.

The changes which occur in proteins and in other biologic macromolecules after SV40 induced or after spontaneously occurring transformations in tissue culture of mouse and other mammalian cells were studied. Dr. Chandrasekaran, together with Mrs. V. W. McFarland found that an ∿55,000 MW cellular protein which is "induced" after SV40 infection of cultured mammalian cells, is also present in embryo primary cells which were not exposed to the SV40 virus. The mouse embryo protein and the "induced" protein in SV40 transformed mouse cells when labelled with ^{35}S-methionine have virtually similar 2D tryptic peptide fingerprints. The monoclonal antibody to the SV40 induced protein precipitates the embryo protein. Both types of proteins are phosphorylated mainly in the serine residues both by $^{32}PO_4$ *in vivo* and also in the immunoprecipitate by ^{32}P ATP. The latter result implies associated specific kinase activity, also observed by other investigators. Mouse, rat and hamster embryo ^{35}S-labelled 55K proteins and the 55K SV40 induced proteins from the same species are structurally identical, but have species divergence approximating the expected evolutionary order and divergence. Portion of the 55K SV40 induced protein was found by biochemical techniques on the cell surface (work with Dr. Luborsky). Immunologic crossreactivities imply that it also may be on the surface of the embryo cells.

All of these make it likely that the 55K protein has (or had) an important, i.e. evolutionary conserved, cellular function. We do not know what this function is, or whether it is important in embryonic differentiation. However, Dr. Chandrasekaran and Mrs. McFarland found a significant decrease during the development of the embryo: the amount of the 55K protein in the primary cells or in the organ culture from 12 day old mouse embryos is high, but decreases to a very low level from 16 day old mouse embryos. Replating midgestation embryo primary cells from mouse, rat and hamster leads to a great decrease in the 55K protein.

Thus, the established cells lines or clones, which are mainly fibroblasts, have only very low amount of the protein, be they normal cells or highly tumorigenic spontaneously transformed (sarcomagenic) cells. Dr. Chandrasekaran found that in both the spontaneously transformed cells and in normal cells, the half life of the protein is very low ($\leq$60 min.). In SV40 transformed cells the SV40 coded T antigen appears to stabilize the 55K protein by direct interaction. However, he cannot find signs of any interacting proteins in those cells which are not transformed by SV40 (embryo primaries, mouse embryonal carcinoma cells, L cells and neuroblastoma cells) but contain substantial amounts of the 55K protein. Dr. Chandrasekaran and Mrs. McFarland could not find any correlation of the amounts of the cellular 55K protein with the cell division rate, or a decrease in the amounts after contact inhibition of cell division in established cells.

Nevertheless the 55K protein may provide a unique activity for study. This protein together with the SV40 T antigen, appears on the surface of the SV40 transformed cells, as well as in their nuclei (Drs. Luborsky and Chandrasekaran). Thus the 55K cellular protein may represent as it does a "contact point" reaching back to the DNA level in the regulatory pathways in DNA replication and cell division in two systems: the differentiating embryo cells and the SV40 transformed cells. Dr. Luborsky recognized this, and initiated a careful study on the interaction of the 55K protein with DNA, similar to the interaction of the SV40 T antigen with (calf thymus) DNA originally found by Dulbecco. A fraction of the SV40 T antigen and also of the 55K protein, isolated from nuclei or from surface membranes of SV40 transformed cells, were found indeed to interact with calf thymus DNA, as eluted only at elevated pH and salt concentrations. Similar fractions of the 55K proteins are now being obtained from the mouse embryonal carcinoma F9 cells which contain the 55K protein but not the SV40 T antigen. The latter result implies that the 55K protein (from both the nuclei or from the surface membrane) indeed interacts with the cellular DNA directly, not just because through the SV40 T antigen with which it is in complex in the SV40 transformed cells. Dr. Luborsky plans to expand this work to study the midgestation embryo cells. These interactions may open up novel studies on the effect of new type of proteins in regulating cucaryotic cell growth and differentiation.

Much of the work on the 55K proteins in other laboratories is on transformed or tumorigenic cell lines. We do not find a general correlation with tumorigenicity when the spontaneously occurring tumorigenic transformed mouse fibroblast cells (clones) are compared with the normal clonal parent cells, in very carefully matched cell families. We feel that the importance of our discovery is in the finding that the 55K protein is being expressed constitutively in normal embryogenesis, and in the finding that the same cellular gene expression being modulated in many transformation processes such as in SV40 transformation. This can lead to the convergence of two fields of investigations: There is now a potential avenue of applying molecular biology techniques and reagents which are so well defined in tumor virology, to some yet undefined changes which occur in

in embryogenesis.

There was considerable research use of the well defined mouse cell families developed painstakingly for genetic and biochemical studies during the last several years (Mora and McFarland). Dr. Winterbourne, who carried out a very careful study on the glycosoaminoglycans on the surface of cells, found that both spontaneous transformations and the SV40 transformations of the same parent clonal mouse cell, which are both very rare events, result in a highly specific change in the biosynthetic pathways: the reduction in the 6-O-sulfate glucosamine in a particular region of the heparan sulfate molecules. Careful analysis of our genetic and biologic data with his biochemical findings led us to conclude that this common biochemical change could not just happen by "chance", it may have a common correlate in the increase in the synthesis of cellular DNA accompanied by increased activities of the appropriate enzymes in both spontaneously occurring and by SV40 induced cellular transformations. Dr. Winterbourne returned to England, but continues these studies on our cells.

We also have an unparalleled collection of spontaneously transformed clonal mouse cells in closed families with careful control on the pedigree and on the selection factors both in vivo and in vitro, (clones obtained with V. McFarland and L. Waters). We think this allows us to set up a unique and rational approach for the analysis of the seemingly intractable problem of "spontaneous" (chance) transformation. One interesting biologic finding shows that the highly tumorigenic (sarcomagenic) clonal mouse cells which attained a stable heritable tumorigenic phenotype in a low frequency ($1/10^6$) apparently by a mutational event, as a rule do not grow without anchorage in a viscous medium in vitro. Thus while cells which grow well without anchorage are generally tumorigenic, the opposite is not true. Obviously, for in vivo tumorigenicity (either in the syngeneic or in the nude mouse) further factors are required in the in vivo interaction of these cells with the host, which do show up in the anchorage independent in vitro cell growth experiments. Our cells therefore appear also useful to study such in vivo factors, both in the syngeneic and the nude mouse.

In further characterization of the well pedigreed families of normal mouse cells, or cells transformed spontaneously or with SV40, we find that spontaneous transformation (cf. by somatic mutation) is sufficient to explain the acquisition of cellular tumorigenicity of the SV40 transformed mouse fibroblasts. There is no binding correlation between cell growth in viscous medium, of tumorigenicity in vivo in both syngeneic and in nude mouse and of the SV40 expression in the mouse fibroblast cell families we have investigated. This confirms our previous observation that on the balance the phenotypic changes pertaining to in vivo properties of cells attributed to SV40 early gene coded T antigen are predominantly expressed as cell surface antigens, facilitating immunologic recognition and rejection of the cells in the immunologically competent syngeneic mouse. Our results underline the growing recognition that for the acquisition of tumorigenic potential the transformation by SV40 is not a sufficient cause, and may not even be a (crucial) contributory event in the mouse (or rat) cells. Beside the above mentioned work and that of Dr. Winterbourne, we are exploiting our collection of clonal cells in numerous collaborative studies in this Country and abroad (see Section II and III of Z01 CB 05526-13).

We plan now to investigate the cellular DNA changes in the spontaneous transformations by transfection of normal parent clones with restriction endonuclease treated DNA from the derivative tumor clones.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05097-02 IP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure of Thyroglobulin and Interaction with Membrane Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sidney Shifrin	Chemist	IP	NCI
Other:	L. D. Kohn	Medical Officer	LBP	NIAMDD
	J. E. Rall	Director		NIAMDD
	Paolo Vitti	Visiting Scientist		NIAMDD
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	Mark Willingham	Chemist	LMBGY	NCI
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COOPERATING UNITS (if any) Frank Maley, N.Y. State Dept. of Health, Albany, N.Y.; William Valente, University of Maryland; Eduardo Consiglio, Salvatore Aloj, Gaetano Salvatore, Paolo Lacetti, University of Naples; Roy Sundick, Wayne State University

LAB/BRANCH
Immunology Program

SECTION
Macromolecular Biology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☑ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to examine the structures of thyroglobulin, thyrotropin (TSH), interferon, and cholera toxins in order to elucidate the structures which are necessary for interaction with receptors on thyroid membranes.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The purpose of these studies is to determine the chemical nature of the structures of several thyroid hormones, toxins and interferon which participate in binding, internalization and biodegradation by thyroid cells.

Methods Employed: Affinity chromatography, two dimensional gel electrophoresis, chemical modification of proteins and receptors, chemical modification and isolation of glycopeptides from glycoprotein hormones, preparation and characterization of monoclonal antibodies, purification of enzymes involved in ADP-ribosylation, ultraviolet absorption spectroscopy, fluorescence, circular dichroism, light scattering, radioautography.

Major Findings: I. Bovine thyroglobulin binds to the thyroglobulin receptor via tyrosyl residues since chemical modification of tyrosyl residues either by nitration with tetranitromethane or by O-acetylation with N-acetylimidazole markedly reduces binding of 19S thyroglobulin to bovine thyroid membranes. 27S thyroglobulin can be prepared in vitro by removing sialic acid and galactose from the B carbohydrate chain of 19S thyroglobulin using neuraminidase and β-galactosidase, respectively. This transformation from 19S to 27S is quantitative if N-acetylglucosamine is also removed from the B chain by N-acetylglucosaminidase. The transformation is facilitated with thyroglobulin containing a high iodine content. The 27S material prepared in vitro has many of the properties also found with 27S iodoprotein made in vivo. The fluorescence is quenched probably because of a nearby iodotyrosine; the lysyl residues are more reactive with trinitrobenzyne sulfonic acid. The 27S thyroglobulin is more readily dissociated by urea and by succinylation than 19S thyroglobulin. The 27S material binds to thyroid membranes to a greater extent than 19S thyroglobulin. Chemical modification of the tyrosyl residues of 27S material with either tetranitromethane or N-acetylimidazole does not interfere with binding to thyroid membranes. The 27S material binds to a different site than 19S thyroglobulin. The 27S material also inhibits binding of TSH. (With Kohn (Z01 AM-23960 LBP, NIAMDD), Rall, Consiglio, Salvatore, Sundick.)

II. TSH inhibits the binding of interferon to functional thyroid cells in culture (FRT_L) and less so to non-functional cells (FRT). There appears to be some common sequences in the structures of TSH, cholera toxin and interferon. Synthetic interferon (22-166) prepared by Drs. Mark Smith and Christian B. Anfinsen binds to functional thyroid cells in culture.

a) Interferon - IF - FRT_L cells have been found to be exquisitely sensitive to the presence of interferon. The cells respond to the interferon within one hour after the addition of the antiviral agent and produce 3',5'-oligo A synthetase. We have found that the preincubation of the cells with TSH prevents the action of interferon on FRT_L cells but has a smaller effect on FRT (non-functional) cells. (With Friedman, Smith and Anfinsen (Z01 AM-25000 LCB, NIAMDD.)

Smith and Anfinsen have chemically synthesized human IF and some variations of human interferon in which phenylalanine has been substituted for tryptophan. These peptides have been radiolabelled by a variety of techniques and their ability to bind to FRT_L membranes has been examined. One of the larger synthetic fragments has been found to bind to a significant extent to these

membranes and the potential biological function of the material is being investigated.

III. TSH Stimulated ADP-ribosyl Transferase in Thyroid Membranes. A variety of TSH derivatives have been prepared in order to examine the nature of the functional groups of this hormone which are operative in this enzymatic reaction. Also cholera toxin derivatives are being examined.

a) Thyroglobulin isolated from chickens which are hyperthyroid or have autoimmune thyroiditis differ in the distribution of 12S, 19S and 27S forms. Thyroglobulin isolated from the OS chickens which have autoimmune thyroiditis is predominantly 12S and binds very poorly to thyroid membranes. Thyroglobulin isolated from hyperthyroid (CS) chickens is predominantly 19S although this form is unstable and rapidly degrades to 12S which binds poorly. Normal chicken thyroglobulin contains 12S and 19S forms. We are examining the reasons for the presence of particular forms in these genetic strains. (With Kohn (Z01 AM-23960 LBP, NIAMDD) and Sundick).

b) Chemical Modification of the Amino Acid Side Chains. Bovine thyroglobulin has been modified by succinylation, trinitrophenylation, nitration and O-acetylation in a study of the effect of these modifications on the structure of the thyroglobulin and on the binding to receptors.

Low degrees of succinylation do not affect the state of aggregation of 19S thyroglobulin but increase binding to thyroid membranes. As the extent of succinylation increases the 19S from dissociates into 12S subunits and the binding to thyroid membranes increases.

Trinitrophenylation of lysyl residues does not affect subunit interactions but does increase binding to thyroid membranes.

Chemical modification of tyrosyl residues either by nitration with tetranitromethane (TNM) or by O-acetylation using N-acetylimidazole did not produce dissociation of the 19S from but markedly reduced binding to the thyroid membrane. Removal of the O-acetyl group with hydroxylanine allowed ^{125}I-thyroglobulin to bind to the membrane.

c) Modification of the Oligosaccharide Moiety. Removal of the terminal sialic acid residue with neuraminidase does not affect the state of aggregation of 19S Tg. However, when the penultimate galactose residue is removed with β-galactosidase from Aspergillus, nearly 30% of the protein has aggregated to the 27S form. This 27S material prepared in vitro has many of the physicochemical properties found with 27S iodoprotein made by the thyroid gland in vivo. For example, the fluorescence of thyroglobulin (19S) is quenched as a result of the association, the emission maximum indicates that the tryptophan residues are in a more hydrophilic environment.

The 27S material prepared either way binds to a much greater extent to thyroid membranes than does the 19S form. While succinylation or trinitrophenylation markedly enhances binding to thyroid membranes, modification of the tyrosyl residues has no effect on binding.

The 19S Tg and 27S form bind to different binding sites on the membrane. The rate of internalization of 19S versus 27S by FRT (non-functional) and FRT_L (functional) cell is currently under investigation.

The removal of the third sugar in the B chain of thyroglobulin (N-acetylglucosamine) by the action of β-N-acetylglucosaminidase from Aspergillus caused more 19S thyroglobulin to aggregate to the 27S form than had occurred after removal of the penultimate galactose residue.

The role of the sugar moieties in binding to thyroid membranes is being investigated in collaboration with Dr. Frank Maley who is isolating the sugar moieties by the use of Endoglycosidase H. Independently, we digested the polypeptide backbone of 19S thyroglobulin with proteolytic enzymes and obtained a crude mixture of the oligosaccharides and some oligopeptides. This crude mixture inhibits binding of ^{125}I-thyroglobulin to thyroid membranes. Upon further separation of the A and B chains over a Dowex column, the inhibitory fragment has been lost.

d) Presence of Phosphomannose. We observed that either mannose-1-phosphate or mannose-6-phosphate can inhibit the binding of Tg to thyroid membranes at pH 5 in the absence of added calcium. Similarly monophosphopentamannose is especially effective as an inhibitor. These results suggested that binding to the membranes may occur via a mannose phosphate residue on the thyroglobulin molecule. All attempts to detect mannose phosphate directly by TFA hydrolysis followed by fluorometric determination with glucose-6-phosphate dihydro ginase, phosphoglucosisomerase and phosphomannoisomerase have failed. However, by using radiolabelled sugar precursors together with ^{32}P, it is now clear that thyroglobulin contains a diester of N-acetylglucosamine and mannose. Whether this grouping is part of the binding iste is not clear. However, the role of this functional group in directing thyroglobulin to the lysosome where it will be degraded to produce the thyroid hormones, triiodothyronine and thyroxine, is under investigation. (With Kohn (Z01 AM-23960 LBP, NIAMDD), Salvatore and Consiglio).

e) Attempts to Isolate the TSH Receptor. Yavin and Valente have prepared monoclonal antibodies against the TSH receptor which will inhibit the binding of ^{125}I-TSH to thyroid membranes. This monoclonal antibody will also activate adenylate cyclase in thyroid membranes. An attempt is being made to prepare an affinity column with the monoclonal antibody, extract thyroid membranes with non-ionic detergents (Triton-X) and isolate the receptor in a manner similar to that described by Ashwell for the hepatic asialoglycoprotein receptor. (With Yavin, Valente, Lacetti and Kohn (Z01 AM-23960 LBP, NIAMDD)).

f) Uptake of Tg and Modified Tg's by Fibroblasts. 19S thyroglobulin and 27S thyroglobulin have been labelled with rhodamine in an attempt to follow their route of uptake and internalization in fibroblasts. Thyroglobulin which was modified with monophosphopentamannose was found to inhibit the uptake of L-iduronidase (Rachel Meyerowitz, NIAMDD) which is taken up via the mannose phosphate receptor in fibroblasts. (With Pastan and Willingham (Z01 CB-08712 LMB, NCI).

Normal human fibroblasts did not appear to have sufficient receptors specific for this sugar phosphate and other types are under investigation -

particularly Chinese hamster cells.

g) ADP-Ribosyltransferase. Cholera toxin possesses ADP-ribosyltransferase activity and although TSH does not have this activity, it can stimulate the ADP-ribosyltransferase of thyroid membranes. There is evidence (Vitti and Kohn (Z01 AM-23960 LBP, NIAMDD) that TSH itself is ADP-robylated in addition to the ribosylation of some membrane compoents including the G regulatory protein.

I have prepared a large number of derivatives of TSH and of cholera toxin in an attempt to determine the ADP-ribosyltransferase site on cholera toxin and to determine the site of ADP-ribosylation on TSH.

Significance to Biomedical Research and the Program of the Institute: There are a large number of thyroid tumors which affect the structure of thyroglobulin, i.e., its biosynthesis as well as its biodegradation. Our studies are directed at elucidating the nature of the binding site on thyroid membranes and the structural features of Tg required for binding and degradation.

Proposed Course of the Project: Monoclonal antibodies against TSH have been prepared and we plan to use it in the isolation of the TSH receptor. Bovine thyroglobulin is being cleaved by enzymatic and by chemical methods in an attempt to determine that portion of the molecules which is involved in binding to the thyroid membrane receptor. We are also purifying ADP-ribosyltransferase from thyroids in an effort to determine how this enzyme provides a mechanism for control of cellular processes.

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Consiglio, E., Shifrin, S., Yavin, Z., Ambesi-Impiombato, F. S., Rall, J. E., Salvatore, G., and Kohn, L. D.: Thyroglobulin interactions with thyroid membranes. Relationship between receptor recognition of N-acetylglucosamine residues and the iodine content of thyroglobulin preparations. J. Biol. Chem., 1981, in press.

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DeWolf, M. J. S., Yavin, E., Yavin, Z., Consiglio, E., Vitti, P., Shifrin, S., Epstein, M., Gill, D. L., Grollman, E. F., Lee, G., and Kohn, L. D.: Studies on the Mechanism of Action of Thyrotropin. In Albertini, E. (Ed.): Radioimmunoassay of Hormones, Proteins and Enzymes. Proceedings of the International Symposium, Gardone Rivera, Italy, May 8-10, 1980, International Congress Series

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB-05545-01 IP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Expression of Cellular Antigens in Transformed Cells and in Embryonic Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	V. W. McFarland	Chemist	IP	NCI
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TOTAL MANYEARS: 2.5	PROFESSIONAL: 1.5	OTHER: 1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to further identify, isolate and characterize an SV40 induced 55,000 MW cellular protein in embryonic cells. Primary cells prepared from midgestation mouse, rat and hamster embryos expressed the 55,000 dalton protein constitutively without SV40 infection. The 2D tryptic peptide map showed that this protein is similar to SV40 induced 55K protein and is conserved evolutionarily in both embryonic and in SV40 transformed cells. The embryo protein is a phosphoprotein. The amount of the protein in embryo was found to be half that of SV40 transformed cells and its presence correlated with the age of the embryo (in the case of mouse), maximum being at 12 day and minimum at 16 day. The amount of the protein decreased also with the tissue culture passage of embryo primary cells. The expression of the protein did not correlate with tumorigenic transformation in established mouse cells.

Project Description

Objectives: To determine whether the SV40 induced 55K cellular protein is expressed in embryonic cells and in spontaneously transformed tumorigenic cells and also to study the location of 55K protein in SV40 transformed mouse cells.

Methods Employed: Preparation of primary, secondary and tertiary cultures from embryos of different periods of gestation. Radioactive labelling, immunoprecipitation gel electrophoresis, electrofocussing, thin layer chromatography and fluorography. Cell growth dynamics in culture.

Major Findings: A specific 55,000 dalton protein was shown to be present in SV40 transformed cells in addition to SV40 coded large T and small t antigens. This 55K protein is not encoded by SV40 DNA but it is of cellular origin. Studies on tryptic peptides revealed that this is an evolutionarily conserved protein. The same or similar protein was detected in a variety of transformed cells induced by various agents such as meth A, x-ray and RNA tumor virus. The same or similar protein was also detected in embryonal carcinoma cell without SV40 infection. The objective of our study was then to find out whether the expression of this protein correlates with embryonic development and/or with tumorigenic transformation. The location of this protein in SV40 transformed cells was also studied, to find some clue pertaining to its possible function.

A) 1. 55K Protein is an Embryo Protein. Mouse embryos at various post implantation stages (10-16 days) were investigated and compared. Primary cultures from 10 and 12 day old mouse embryo showed the presence of specifically immunoprecipitable 55K protein. The presence of this protein was further confirmed by

a. immunoprecipitation of 55K protein from primary embryo cultures with a monoclonal antiserum prepared against SV40 induced 55K protein,

b. the comparison of ^{35}S-methionine labelled tryptic peptides of embryo 55K and SV40 induced 55K proteins indicated similar tryptic peptide pattern (in collaboration with Dr. D. Simmons),

c. the embryo 55K protein was labelled with ^{32}P indicating the protein to be a phosphoprotein similar to SV40 induced 55K.

2. Correlation of 55K with Embryo Age and Tissue Culture Transfer. The presence and the amount of the 55K protein was then investigated and quantitated from different day embryos. The amounts were then compared with the amount of SV40 induced 55K protein. The amount of the protein present in 12 day old embryo primary cells was found to be about 50% compared to SV40 induced 55K. The amount of the protein in older embryos decreased with the age of the embryo, as shown - 13 day - 40%, 14 day - 25%, 15 day - 7.1% and 16 day - 2.6%. Thus the expression of the protein correlated with the age of the embryo.

Attempts were made to identify the organs which are responsible for the synthesis of this protein in embryos. In collaboration with Dr. M. Dziadek we dissected and labelled various organs from 12 day and 16 day mouse embryos. The results showed that in 12 day old mouse embryo the following organs were found to possess the 55K protein - liver, brain, heart, carcass and possibly visceral yolk sac whereas the same organs isolated from 16 day old mouse embryo did not possess the protein. The expression of this protein was studied upon

tissue culture transfer (t.c.t.) of the embryo primary cells. The 12 day mouse embryo primary cells which possessed the protein when trypsinized and replated were found not to contain the protein (the first t.c.t.). Similar results were obtained during the second t.c.t. also. Thus there could be a tissue culture selection against these cells which synthesize the 55K protein.

3. Presence of 55K Protein in Murine Embryos. The presence of the protein in embryos was further confirmed by studying the embryos of other species, i.e. rat and hamster. Immunoprecipitation with the SV40 T serum and with monoclonal serum indicated the presence of 55K protein in 14 day old rat and 10 day old hamster embryo primary cells. The amount of the protein precipitated was found to be 60% in hamster embryos and 45% in rat embryos compared to SV40 induced 55K protein. The first tissue culture transfer cells derived from hamster embryo primary cells synthesized about 10% of 55K whereas the second t.c.t. cells did not show the presence of 55K. On the other hand the first t.c.t. cells from rat embryo primary cells did not show the protein. Thus the hamster embryo primary cells which synthesize maximum amounts seem to retain some cells after first t.c.t. and therefore continue to show the presence of 55K protein whereas other embryo primaries did not. The presence in other embryos is further confirmed by 2D tryptic peptide map. The 2D map of hamster embryo is found to be similar to that of 55K from SV40 transformed hamster cells. Thus the embryo 55K protein also seems to be evolutionarily conserved as is the SV40 induced 55K.

4. Correlation of 55K Protein with Differentiation of EC Cells. Embryonic carcinoma cells (F9 and OTT6050) were tested and found to express the 55K protein. The methionine containing tryptic peptides was found to be similar to SV40 induced 55K protein. The EC cells were then subjected to differentiation by treatment with retinoic acid and the expression of 55K protein in such cells was then studied. The results indicated that the amount of the protein decreased with increased differentiation and was not present in fully differentiated (parietal endoderm) EC cells. On the other hand established parietal yolk sac cell line such as PYS-2 were found to express the same protein. Thus it remains to be seen whether the detection of 55K protein could be correlated with expression or differentiation of any particular cell type.

B) 1. Expression of 55K Protein is not a General Correlate of Cellular Tumorigenicity. We present here a summary of experiments on three families (two AL/N and one Balb/c strain) of mouse clonal cell lines, and derivative tumor lines with respect to the expression of 55K protein.

The first family of cells originated from a spontaneously transformed highly malignant (median tumorigenic dose, $TD_{50}=10^2$ cell/mouse) AL/N mouse embryo cell clone 104C. We have shown previously that after SV40 transformation the tumorigenicity of 104C cells in immunologically competent syngeneic mice becomes lower ($TD_{50}=10^4$), apparently because of the expression of the SV40 T and transplantation antigens. One such "daughter" cell, the SV40 T antigen positive clone *106CSC was shown to contain 1 copy number equivalent of SV40 DNA per cell DNA. When we injected *106CSC cells into the syngeneic mouse, a negative immunologic selection resulted in T antigen negative revertant derivative tumor lines and clones that originated from a rare cell(s) through some DNA rearrangement. These T antigen negative revertants (i.e., the 124, 127, 128 CSCT

and 134SCTC cells) retained any "late" SV40 DNA sequences, and probably also a region (Taq 1 - Hha 1, 0.73 - 0.55 map units) which includes the sequence coding for small t antigen (0.65 - 0.55 map units). In the tumor lines and clones (128, 127, 124CSCT and 134SCTC) negative for large T antigen there was no specific 55K MW protein band detectable, just as in the distant parent 104C cell before the SV40 transformation, while the proximal parent T antigen positive *106CSC cell possessed the 55K protein. Thus the presence of one copy number equivalent of early SV40 DNA encompassing sequences coding for large T (but not necessarily for small t) antigen, and the resulting synthesis of SV40 large T antigen, is required and is sufficient in this family of mouse embryo fibroblast cells for the production of the 55K cellular protein. Our results with the T antigen revertant cells extend the findings of others by demonstrating clearly that the continued presence of the A gene and synthesis of large T antigen is required for the maintenance and production of the 55K cellular protein. In SV40 transformed cells large T antigen is shown to form a complex with 55K protein and therefore the precipitation of 55K could be due to this complex formation. However, it must be noted that the serum from SV40 tumor bearing hamsters, used thorughout these experiments, precipitated the 55K cellular protein alone from the F9 EC cells and from the embryo cells. After extracts of labelled SV40 transformed cells were immunoprecipitated and separated by gel electrophoresis, separate bands of the gel at the 55K and 94K region were cut out, eluted, re-precipitated by the tumor serum, and re-electrophoresed. The appropriate 55K or the 94K proteins immunoprecipitated. Thus the serum from SV40 tumor bearing hamster used by us contained specific antibodies against the 55K cellular protein. When excess amounts of unlabelled extracts of *106CS cells, which contain T antigen were mixed with the extracts of ^{35}S-methionine labelled 124CSCT cells, there was no more radioactivity precipitated in the 55K region with the SV40 hamster tumor serum than with the control normal serum. This could mean either that there was no complex formation in mixed extracts between the large T antigen of *106CSC cells and the 55K protein of labelled 124CSCT cells, or that the 55K protein is not present in the extract of labelled 124CSCT cells. Since the serum from SV40 tumor bearing hamster used is capable of precipitating large T antigen from SV40 transformed cells, and independently the 55K cellular protein from F9 and embryo cells in the same extraction buffer as in the mixing experiment, and in buffers with other detergents in the separation and re-precipitation experiments. These results are interpreted to mean that the highly malignant ($TD_{50} \leq 10^2$) 124CSCT and all the other T antigen negative cell lines do not synthesize (detectable amounts of) 55K cellular protein.

The second family of cells studied originated from a clone (210C) of AL/N mouse fibroblast cells which possessed very low tumorigenicity ($TD_{50}=10^{6.5}$). However, when injecting 10^7 210C cloned cells, tumor lines of high tumorigenicity ($TD_{50}<10^2$) can be obtained, such as the 219CT. The simplest explanation is that in 210C cells, variant (mutant) "spontaneously" transformed highly tumorigenic cells(s) arise at low frequency, and these cells are selected for tumorigenicity by the *in vivo* passage. The highly tumorigenic 219CT cells possess no 55K MW cellular protein. When the 210C cells were infected with SV40 and immediately recloned, the derivative T antigen negative daughter clone 213CSC had no 55K MW cellular protein, while the independently transformed SV40 T antigen positive daughter clones *214CSC and *215CSC both had the 55K cellular protein. Note that the transformation of 210C with SV40 resulted in the T antigen positive

*214CSC and *215CSC clones which have similar low tumorigenicity as the 210C. From 10^7 *215CSC cells the tumor lines obtained from separate mice all had high tumorigenicity ($TD_{50}10^2$-10^3), irrespective of whether they were T antigen positive (*221CSCT, *222CSCT, and *224CSCT) or T antigen negative (223CSCT); the 223CSCT being probably a similar revertant to those discussed in the first cell family. Only the SV40 positive clones were shown to contain 55K protein.

In the third cell family, derived from a Balb/c strain tumor, the highest relative tumorigenicity was the property of the parent line 301 T, which was the only SV40 T antigen negative line and that was also the only line that did not contain the 55K protein. Thus all the spontaneously transformed malignant sarcoma cells tested from AL/N and Balb/c mice did not contain the 55K cellular protein. The tumorigenic and non tumorigenic cell lines synthesize small amounts of 55K (about 2%) when compared to SV40 transformed cells. We conclude that the presence (synthesis) of the 55K cellular protein is not a correlate of spontaneously arising tumorigenicity (malignant sarcomas) in established mouse fibroblast cells, but it is an absolute correlate of SV40 infection and transformation.

2. Half Life Determination. The absence of detection of this protein in tumorigenic cell lines could be due to differential turnover of this protein in these cells and the SV40 virus transformed cell lines. To study this the cells were labelled for a short period of time (30 min.) and then chased for various periods of time. The cell extracts were then immunoprecipitated and the 55K band was quantitated either by direct counting or by integrating the peak area. The results showed that during a 30 min. pulse label all the non-tumorigenic and tumorigenic non-SV40 transformed, as well as SV40 transformed cell lines showed the presence of 55K protein but the amount of much lower in the non-transformed cell lines compared to SV40 transformed cells. The half life of the protein was also found to be different. The 55K in non-SV40 transformed tumorigenic and non-tumorigenic cell lines had a half life of about 60 minutes, whereas the 55K in SV40 transformed cells had about 300 minutes.

C. Subcellular Distribution and Heat Stability of 55K Protein in SV40 Transformed Cells. The subcellular distribution of this protein was then studied in SV40 transformed cells to locate the 55K protein. Our initial studies on subcellular fractionation indicated the presence of 55K protein both on nucleus and on the surface of SV40 transformed cells. We utilized the following biochemical studies to confirm these observations.

a) Trypsin treatment of metabolically labelled intact SV40 transformed cells to determine the proteins released from the surface of these cells showed the presence of both large T and 55K on the surface.

b) Lactoperoxidase catalyzed iodination from the outside showed that both large Tag and 55K were iodinated by this method and these proteins were then digested preferentially by trypsin.

c) Large T antigen in cell extracts were found to be less heat stable compared to 55K protein and the 55K protein was precipitated in these cell extracts without the precipitation of large T antigen. Such heat treated cells were then shown to protect mice when immunized against SV40 induced tumor development.

Significance to Biomedical Research and the Program of the Institute: Re-expression of embryonic antigens in transformed cells have been used in immunological protection studies against tumor development. Analysis of the identification of these proteins would provide more insight into the mechanism of transformation and the phase specific expression of this protein could be used at the molecular level to study embryonic differentiation.

Proposed Course of the Project: Attempts will be made to determine whether any particular cell type from embryonic cells could be isolated that would synthesize the protein constitutively or to find out whether this protein would serve as a marker of differentiation of embryonic cells. In this regard, in collaboration with Drs. Solter and Edidin, we plan to detect this protein in pre-implantation embryos. We also plan to use the monoclonal antibodies against 55K to detect the presence of this protein in spontaneous transformed tumorigenic cells. The nature of phosphorylated amino acid in the 55K protein from both SV40 transformed cells and embryo cells will be studied in an attempt to find its biochemical role in transformation and embryonic development.

Publications:

Mora, P. T., Chandrasekaran, K., and McFarland, V. W.: An embryo protein induced by SV40 virus transformation of mouse cells. Nature 288: 722-723, 1980.

Chandrasekaran, K., Winterbourne, D. J., Luborsky, S. W., and Mora, P. T.: Surface proteins of SV40 transformed cells. Int. J. Cancer 27: 397-407, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05526-13 IP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Cell Surface Changes in Spontaneously or SV40 Transformed Mouse Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	V. W. McFarland	Chemist	IP	NCI
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	.5	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the cell surface membrane, how it may change in transformation to malignancy, either by spontaneous induction, or as induced by SV40 virus; to study the nature of biochemical and biologic changes associated with the cells being transformed by either SV40 or by spontaneous event(s).

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To elucidate certain biochemical and biological changes both in the cell surface and in the control mechanisms involved in cell division, which occur during transformation to malignancy in mouse cells.

Methods Employed: Labelling glycoproteins, glycosaminoglycans and glycolipids, proteins and nucleic acids with radioactive precursors during cell growth in culture. Novel fractionation techniques for isolation of (labelled) subcellular components, including cell surface membranes and macromolecules associated with surface membranes. Isolation and characterization procedures include thin-layer and column chromatography, gel electrophoresis, electrofocusing and autoradiography of gel slabs. Analytical and preparative ultracentrifuge studies. Enzymological techniques pertinent to intermediary metabolism. Cell growth dynamics in culture, and also in vivo, in syngeneic and in nude mice.

Major Findings: I. Analysis of Glycosaminoglycan Metabolism. We have previously shown that, after infection of a "parent" cloned mouse cell (210C) by SV40, the metabolism of heparan sulfate is altered only in the subclones which possess SV40 DNA and express the SV40 T-antigens, but not in an untransformed T-antigen negative "sister" clone. Such changes in heparan sulfate were first reported by others in SV40-transformed 3T3 mass cell lines selected for their ability to form foci in cell culture. Thus, the altered metabolism was detected consistently in those cells which express the SV40 T-antigens, the proteins thought to be responsible in transformed cells for stimulation of DNA synthesis and for loss of growth control.

As an alternative to the virus transformation model, we have isolated inter alia two highly tumorigenic tumor cell lines: 219CT and 220CT by in vivo selection from two independent "normal" clones (210C and 216C respectively) of AL/N mouse fibroblasts which possess very low tumorigenicity. Because these tumor cell lines were isolated from tumors induced in the syngeneic mouse by injecting 10^7 or 10^6 cloned cells, and because the tumor lines possess heritably very high tumorigenicity (median tumorigenic dose $<10^2$ cells/mouse), the simplest explanation is that they represent variants (possibly mutants) which arose spontaneously by a chance event at a very low frequency, and which must have been selected out from the clones on the basis of their high tumorigenicity. We reasoned that, if there is a specific biochemical difference which shows up in between at least two spontaneously transformed tumor cell lines and their respective parent clones, which is also the same between the parent and the SV40-transformed daughter clones mentioned above, this is unlikely to be just a coincidence, as both models of transformation select very rare transformed cells by presumably quite different mechanisms, but most likely it reflects basic and common biochemical alterations in both types of transformation events.

First we confirmed that the same specific alteration in anion exchange properties of glycosamine and glycosaminoglycans as was previously reported for SV40 transformed subclones, was found in both 219CT and 220CT. One tumor line (219CT) and one SV40 transformed subclone (215CSC) were then compared with their common parent clone (210C). The preliminary finding was discouraging: cellulose acetate electrophoresis at pH 1.0 showed that 215CSC heparan sulfate had a slight overall decrease in sulfation compared with heparan sulfate from 210C, however no

gross difference could be detected between heparan sulfate from 219CT and 210C. However, analysis of the products of deaminative cleavage of heparan sulfate by nitrous acid under conditions where cleavage occurs quantitatively at N-sulfated glucosamine residues showed that although heparan sulfate from the three cell lines gave similar yields of O-sulfated disaccharides, both 215CSC and 219CT had only about half as many O-sulfate residues in higher molecular weight oligosaccharides compared to heparan sulfate from 210C. Then an enzymatic degradation of heparan sulfate with a mixture of enzymes from F. heparinum showed that heparan sulfate from 215CSC and 219CT had 30% fewer glucosamine residues bearing 6-O-sulfate groups. This is a specific reduction in 6-O-sulfate glucosamine residues which occurs in regions of the chain containing relatively few sulfate groups. It became clear that certain sequences of charged groups present in heparan sulfate from 210C will be found only rarely in heparan sulfate from 215CSC and 219CT, and that this very specific biochemical change is common to both the SV40 transformation and to the spontaneous transformation.

The biochemical findings have been correlated with the biological properties. It should be considered that the altered metabolism of heparan sulfate is related to the ability to grow to high cell densities, as SV40 transformants such as SV3T3 are selected for this ability, and the SV40-transformed cells studied here, although isolated without bias to their growth properties, also were found to have high saturation densities. However, the altered anion exchange property was also observed in the tumor cell line 220CT compared to its parent 216C even though the cell lines have been found to possess similar high saturation densities. Furthermore, the cell growth properties in culture did not correlate with tumorigenicity. This was particularly true for the plating efficiency in methocel (anchorage independent growth) which is generally considered to correlate more closely with tumorigenicity than other cell parameters, and yet this growth was very low for both spontaneously transformed highly tumorigenic tumor cell lines. In addition, we know from our earlier work that transformation by SV40 does not necessarily result in increased tumorigenicity in mouse cells, as the SV40-coded T and tumor-specific transplantation antigen on the cell surface causes recognition and rejection of cells in the syngeneic mouse. However, after SV40 infection of nonpermissive cells, there is in coincidence with the expression of T-antigens an invariable increase in the synthesis of cellular DNA accompanied by increased activities of the appropriate enzymes. Of course it is commonly thought that any tumorigenic transformation must be associated with loss of control of DNA synthesis. Therefore we postulate that it is in this respect, over-riding the normal control of metabolism, a putative element in cell growth control, is somehow connected with the two different forms of "transformations" studied by us.

The biochemical part on this project, the glycosaminoglycan synthesis and related enzymatic changes, the studies on the putative coupled enzymatic changes in DNA biosynthesis have been discontinued in this laboratory since Dr. Winterbourne left. However, we continue to collaborate with Dr. Winterbourne in London, in the selection and the biologic characterization of further (clonal) cells useful for his studies (see below).

II. <u>An Approach to Analyze the "Spontaneous" Transformation of Cells</u>. Obviously there are formidable theoretical odds against a systematic analysis. Yet, during the last five years we carefully developed "families" of closely

related mouse clonal cells, which we believe allows now a rational and unique approach.

The original cause of this work was sheer frustration: with cells obtained from (and selected by) others, many of the biochemical correlates we found (cf. the ganglioside changes with R. Brady) turned out to be more of the function of some tissue culture "selection" process, rather than a general correlate of the cellular tumorigenicity. Incidentally, this probably affects all of the results of biochemists and molecular bologists much more than it is generally recognized, except when conditional (lethal) mutants (cf. ts mutants) of viruses are employed as transforming agents. For a meaningful study of "spontaneous" transformation close and controlled genetic relatedness of cells, statistically significant number of subclones, very careful parallel handling of cells (cf. equal number of passages in culture), and realization and controlled use of the potential selective in vitro and in vivo factor(s) is all essential.

First we selected from an AL/N embryo mass cell line two "parent" clones of mouse fibroblast. One clone (104C) had very high tumorigencity ($TD_{50}<10^2$), the other clone (210C) very low ($TD_{50}=10^{6.4}$ cells/syngeneic mouse representing an essentially normal cell). The 104C cells and its clonal derivatives were and are still most useful in studying the innate spontaneous tumorigenicity, and in the case of SV40 transformed derivative subclones through the expression of the SV40 T and TSTA antigens on the cell surface, the effect of the SV40 coded antigens in functionally opposing the spontaneous tumorigenicity. For example, a series of publications during the last five years led to several important findings: The T antigen of SV40 is the TSTA; T antigen and early SV40 DNA minus revertant cells (clones) can be selected out by transplantation through immunocompetent syngeneic host (this obviously is preceeded by DNA rearrangement which leads to the loss of the early SV40 DNA) etc. The 104C parent clone, in fact had too high degree of DNA "instability" and too high frequency of "spontaneous" transformation to tumorigenicity, thus it was not realistic to use it as an average candidate cell for transformation analyses.

The parent clone 210C (and another clone 220C) were found ideal, however, in their (putative) mutational frequences to tumorigenicity ($1/10^6$ - 10^7). From the 210C clone alone we have isolated 10 independent tumor lines by a single in vivo passage (work with L. Waters): each tumor line has $TD_{50} \approx 10^2$, both in the syngeneic and in nude mice, and this phenotypic property is heritable and stable. By recloning (with V. McFarland) one tumor line we obtained 14 subclones, and we are in the midst of the biologic characterizations. We have preliminary results which encourages us to believe that this family of cells represent an unique collection to study (on a statistically significant level) the biochemical correlates of spontaneous ("chance") transformation on various molecular levels (DNA, RNA, proteins etc.). To exploit these cells fully collaborative work is being set up with many laboratories in this country and abroad (for example transfection of the non-tumorigenic parent 210C with the restriction endonuclease DNA fragments of the derivative tumor clones, etc.).

We wish to bring up one interesting biologic result: the highly tumorigenic mouse clonal cells, as a rule, do not grow without anchorage in viscous medium. Thus while cells which grow well without anchorage are generally tumorigenic, the opposite is not true. Obviously, for in vivo tumor growth further "factors"

are required in the interaction of these cells with the host, which do not show up in the anchorage independent in vitro growth experiments in the viscous medium. Our cells therefore also appear uniquely useful to study such in in vivo factors, both in the syngeneic and in the nude mice.

Third type of cell families, originating from Balb/c strain mouse embryo fibroblast clones, are also under study. Several derivative subclones were obtained (as in the previously described AL/N cell families too) both after spontaneous and after SV40 induced transformations. Numerous clones which were obtained by independent SV40 transformations have been most valuable in the SV40 T antigen studies, and in the SV40 induced 55K protein studies (see below).

III. SV40 Transformation, Cell Biology and Surface Antigen (Protein) Studies. The analysis which led to an absolute correlation of the expression (detectability) of the cellular 55K protein with the presence of functional T antigen in the above referred numerous clonal derivative cell lines has been reported in original papers and in reviews; the recent results are summarized by Dr. Chandrasekaran (Z01 CB 05545-01 IP). The studies on the binding of the 55K protein and of T antigen to DNA, and the presence of these proteins on the cell surface is reported by Dr. Luborsky (Z01 CB 05544-13 IP). The identification of the embryo 55K protein with the SV40 induced 55K protein is in collaboration with D. Simmons, University of Delaware, and the major results are reported in Z01 CB 05545-01 IP, and in Z01 CB 05546-01 IP.

Dr. C. Chang, in Taiwan, has completed a collaborative study on the SV40 T and surface antigen recognition by T cells, he also employs the cells in further collaborative work on (other) tumor specific antigen studies in human biopsies, similar to Dr. Coll in Madrid (see Z01 CB 05545-01 IP).

We supply well characterized clonal cells to numerous investigators in this Country and abroad: Timothy Rose is finishing a doctroal thesis on the SV40 cell surface antigens using our SV40 transformed clones in the Dept. of Molecular Biology in Geneva under Professor R. Weil; Dr. David Lane, at Imperial College, London, studies the interaction of the SV40 T antigen and the 55K cellular protein; Dr. D. Winterbourne at the Dept. of Biochemistry, St. George's Hospital Medical School also at London, studies the glycosoaminoglycan and DNA biosynthetic enzyme changes (see above); Dr. W. Deppert at the University of Ulm, West Germany, studies subcellular distribution of the antigens, etc.

In this laboratory (Drs. Chandrasekaran and Luborsky) used the SV40 transformed clones to detect the SV40 T antigen and also the 55K protein in the cell surface (plasma membrane) enriched subcellular fraction. The accessibility of these proteins on the cell surface was confirmed by radioactive iodination as catalyzed by alctoperoxidase, from the outside of the cell, and also by susceptibility to removal by gentle trypsinization (Dr. Chandrasekaran). Currently Mrs. McFarland is developing fluorescent staining techniques for the detection of SV40 T antigen and for the 55K protein on the cell surface (in part in collaboration with Dr. Edidin, Johns Hopkins, and D. Solter, Wistar Institute).

Using the families of clonal cells, transformed spontaneously and/or by SV40, we find that spontaneous transformation (cf. by somatic mutation) is sufficient to explain the acquisition of cellular tumorigenicity of SV40 transformed mouse

fibroblast. When considering on the balance the in vivo phenotypic changes in mice, the SV40 early gene coded proteins are more "dominant" as cell surface antigens facilitating immunologic recognition and rejection, rather than causing tumorigenic transformation. There is no binding general correlation between cell growth in viscous medium, of tumorigenicity in vivo, and of the SV40 antigen expression, in the mouse fibroblast cells we have investigated. This of course does not mean that the SV40 early gene, its expression, and the role of the T antigen is not a suitable system to study the correlation between certain biochemical controls in cells and their phenotypic tissue culture growth, when such correlations can be made (such as between DNA synthesis and focus formation in culture). Our research, however, emphasizes a growing recognition that for the acquisition of tumorigenic potential the transformation by SV40 is not a sufficient cause, and not even a crucial contributory event. This is apparently true not only in mouse or rat cells, but also in hamster cells (cf. Lewis and Cook, PNAS, 1980).

Significance to Biomedical Research and the Program of the Institute: Studies of biochemical changes in cells, particularly in the cell surface, which relate to acquisition of tumorigenic potential caused by some rare event, such as somatic mutation, and the studies on immunologic recognition and rejection in vivo are of interest, especially when they may lead to understanding of the changes in molecular mechanism which appears to be reducible to heritable changes of control processes on the DNA level.

Proposed Course of the Project: Attempts will be continued to further clarify the relevance of the various changes in cell membrane biochemistry, especially in the heparan and chondroitin sulfates, to spontaneous and to viral induced transformation of cells. We also plan to relate these changes to phenotypic changes in cell immunogenicity and in tumorigenicity in various syngeneic and nude mouse systems. Studies on the molecular level will also include regulatory events which may be due to association of the SV40 A gene product with selective (e.g. nuclear membrane associated) portions of the cellular DNA. We will further study 1) the relationship between SV40 TSTA and the cell surface, 2) control of gene expression for the newly discovered 56K protein and its relation to transformation and its role in embryogenesis, 3) the basis for the interaction of the 56K cellular protein with the various antisera, including monoclonal hybridoma sera, 4) we will also attempt isolation of the transforming allele(s). We hope that the information and emthodology developed in the mouse system will be of use in studies on human tumor cells.

Publications:

Simmons, D. T., Martin, M. A., Mora, P. T., and Chang, C.: Relationship among TAU antigens isolated from various lines of simian virus 40-transformed cells. J. Virol. 34: 650-657, 1980.

Chandrasekaran, K., Winterbourne, D. J., Luborsky, S. W., and Mora, P. T.: Surface proteins of simian virus 40 transformed cells. Int. J. Cancer 27: 397-407, 1981.

Winterbourne, D. J., and Mora, P. T.: Cells selected for high tumorigenicity or transformed by simian virus 40 synthesize heparan sulfate with reduced degree of sulfation. Biol. Chem. 256: 4310-4320, 1981.

Mora, P. T., and Chandrasekaran, K.: Role of SV40 Induced Antigens in Transformation and Rejection of Malignant Mouse Cells, and the Detection of an Embryo Protein. In Manson, L. and Nowotny, A. (Ed.): Biomembranes. New York, Plenum Publishing Corporation, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05546-01 IP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
A Common Protein in Embryonic Differentiation and in Cellular Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. T. Mora	Chief, Macromolecular Biology Section	IP	NCI
Other:	K. Chandrasekaran	Visiting Fellow	IP	NCI
	V. W. McFarland	Chemist	IP	NCI
	C. Parrott	Chemist	IP	NCI
	T. M. Martensen	Staff Fellow	LB	NHLBI

COOPERATING UNITS (if any)
D. Simmons, University of Delaware; E. G. Gurney, University of Utah; M. Dziadek, Kansas State University; J. Coll, Instituto Nacional de Prevision, Madrid, Spain

LAB/BRANCH
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SECTION
Macromolecular Biology Section

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	.5	1.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A specific 55,000 MW phosphoprotein was found in murine embryo cells. This protein is very similar, if not identical, to an SV40 induced protein, isolated earlier from SV40 transformed cultured fibroblasts. The amount of the embryo phosphoprotein is highest in cells taken from midgestation stage mouse embryos. We are studying the biochemical nature and the possible function(s) of this embryonal protein. This (or similar) protein(s) appear in cultured cells which were transformed by various other means including RNA viruses, and also in much lower amounts in normal or spontaneously transformed cells. The presence of protein in high amounts is not a general correlate of cellular tumorigenicity.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To isolate and characterize a specific cellular phosphoprotein of ~55,000 MW which is present in midgestation stage embryo primary cells and also in many cells transformed by various agents, and to find its biochemical function.

Methods Employed: The biochemical and tissue culture methods are outlined in Project Report Z01 CB 05526-13 IP. In addition, complex immunochemical techniques, such as detection of cell (surface) antigens both in cell culture and in situ in embryos with pertinent specific and monoclonal antibodies, dissection of mouse embryos of various ages for organ precursors.

Major Findings: I. Related results are presented in Z01 CB 05545-01 IP by K. Chandrasekaran (it is necessary to summarize these here, to understand the further findings).

1) The isolation and quantitation of the 55K embryo protein from 10-13 day old mouse embryos.

2) Identification of this protein with a monoclonal antibody induced against the 55K protein in SV40 transformed mouse fibroblast cells.

3) The decrease of the embryo protein in mouse embryo primaries, after the embryo was 14 days old or older.

4) Detection and characterization of a similar 55K protein from midgestation age hamster embryos, and demonstration that it is the same protein which is induced in SV40 transformed hamster cells.

5) A loss or decrease of the embryo protein after replating primary cultures.

6) Absence, or presence only in very low amounts of (stable) proteins in normal mouse cloned cells or in spontaneously transformed mouse cells which readily induce fibrosarcoma.

7) A short half-life (fast turnover) of this protein in normal or spontaneously transformed cells.

8) Absolute correlation of the detectability of the protein with the expression of the SV40 T antigen in SV40 transformed clonal cells and in their revertants.

9) Presence of a small amount of this protein on the cell surface of SV40 transformed cells. This is discussed in detail in Z01 CB 05544-13 IP by S. W. Luborsky.

10) Correlation of the 55K protein with the differentiated state of the mouse embryonal carcinoma cells such as the F9 cell line.

II. Further major findings on the 55K cellular protein:

1) The embryo protein is a phosphoprotein. From 14 day old rat embryo primary cultures, labelled with ^{32}P, the 55K protein was specifically precipitated with a monoclonal antibody prepared against the 55K protein from SV40 transformed mouse fibroblast (monoclonal Ab from E. Tucker Gurney, University of Utah). This result was consistent with two of our earlier findings (with D. Simmons, University of Delaware): a) the mouse and rat cells transformed by SV40 produce 55K proteins with many (but not all) ^{35}S-methionine labelled tryptic peptides apparently identical and b) the SV40 induced proteins in a given species and the embryo proteins in the same species give very similar (near identical) peptide fingerprints.

As almost complete identity in the fingerprints were observed between the 55K proteins from SV40 transformed mouse cells and mouse embryo primary cells (which were not exposed to SV40), the easily obtainable 55K phosphoprotein from SV40 transformed mouse cells was chosen for further study. The pure 55K phosphoprotein from the latter cells, labelled by $^{32}PO_4$ appears to possess phosphoserine residues and some phosphothreanine residues, but not detectable amounts of phosphotyrosine residues (with T. Martensen, LB, NHLBI). Recently published results by others (G. Jay et al.) in labelling with $\gamma^{32}P$ ATP (indicating associated phosphokinase activity) in other SV40 transformed mouse cells is consistent with these findings. Concurrently and independently, we also showed an cAMP independent $\gamma^{32}P$ ATP phosphokinase activity associated with the 55K protein (with C. Parrott). We are now involved in determining what type of phosphorylation and phosphokinase activity is present in embryonal carcinoma cells such as in F9 cells, and in embryo primary cells. Since the phosphorylated amino acid is mainly serine, the 55K protein isolated by us is apparently different from the 55K MW phosphokinase intermediate (pK_S) of the protein kinase cascade in Ehrlich ascites membranes which regulates the phosphorylation of Na+ K+-ATPase through the tyrosine residues (recently elucidated biochemically by Racker, and found by other investigators to have pertinence to numerous RNA virus induced transformations of cells). We are exchanging information and reagents with the pioneer groups (E. Racker and D. Baltimore), and are in the midst of experiments (with T. Martensen) in elucidating the biochemical and biologic correlates of the phosphokinase activity of the 55K embryo protein(s).

2) Distribution of the 55K embryo protein during embryogenesis. Attempts are being made to develop detection techniques in various organs and in various phases of the developing embryos. The 13 day or 15 day old mouse embryos were freed of their extraembryonic membranes, and then dissected to provide various organs for labelling with ^{35}S-methionine (with M. Dziadek). The organ cultures of 13 day old mouse brain, liver and carcass showed the presence of the 55K protein, but not of the 15 day old mouse embryos. This is consistent with the drastic decrease of the 55K protein found in embryo primary cultures from 16 day old mice, as compared to the 12 day old embryo primaries (I. 3).

Immunofluorescent methods are being developed to stain in situ sections of (mouse) embryos in different stages of development, using fluoroscein labelled monoclonal antibodies (with V. W. McFarland in collaboration with Davor Solter, Wistar Institute).

3) Cell division rate and the 55K protein. The amount of the 55K protein in embryo primaries was the same when the ^{35}S-methionine labelling was in the presence of 2% or 5% FCS, and the decrease of the 55K protein by embryo age was also unaffected by such variations in serum concentration. Increase in FCS concentration is known to increase cell growth rate, at least in established cell lines. In established clonal normal mouse lines increase in serum concentration (from 2% to 10% FCS) increased somewhat ($\lesssim$10%) of the very low amount of the detectable 55K protein present ($\lesssim$2%, compared to its SV40 transformed derivative clone). Careful cell growth rate measurements and quantitations of the 55K protein are in progress in numerous established normal and transformed cell lines and clones, and also on embryonal carcinoma cells. Up to this time no general correlation is being observed with cell growth rate. It is important to emphasize that our experiments on embryo primaries (as in any other cells) are all in rapidly dividing cells close to their optimal growth rate. Under such conditions the effects of other parameters (such as the age of the embryo, difference between primary and secondary cultures, the presence of SV40 T antigen, transformation (and selection) of certain cells are all much greater (50-100 x higher), than the small effects (<10%) of cell division rate on the amount of the 55K protein in the cells.

4) The detection and possible biologic correlates of the 55K embryo protein in human tumors, and in various (transformed) mouse cell lines.

A) As many (but not all) established human tumor lines were shown by others to contain the 55K protein in well detectable amounts, we embarked on a collaborative screening project on the detectability and quantitation of this protein in freshly established (primary) cells from various human tumor biopsies at a large research hospital in Madrid (Instituto Nacional de Prevision) which has special capacity and competence of this work. The Department of Pathology and Immunology of the hospital has access to large numbers of biopsies of various malignancies, and occasionally of small amounts of (adjacent) apparently normal tissues. The Head of the Cancer Cell Membrane and Immunology Research Section in the Department (Dr. J. Coll) was well trained in the required techniques, beginning at the Rockefeller Institute than at Johns Hopkins and finally in our laboratory: Dr. Coll was the first researcher in this laboratory who established techniques and published careful PAGE analyses of "Metabolically labelled cell membrane proteins in spontaneously and in SV40 virus transformed mouse fibroblasts" (Coll, Luborsky and Mora, Biochemistry 16: 3169, 1977). Now we are supplying Dr. Coll with the specific antibodies necessary for the detection of the 55K protein, and with information on optimizing quantitation of the 55K protein. As yet this collaborative project is in too early a stage to say whether there is any utility to follow the quantitation of this new (class of) embryo "antigen(s)" for diagnosis of any human malignancies.

B) More careful analysis of the question whether the presence of (stable) 55K protein in large amounts is a correlate or not of cellular tumorigenicity is being carried out in our laboratory using newly established clonal mouse cell lines with careful pedigree and close familiar relationships. In mouse fibroblasts from three "families" of cells no correlation was detectable with the TD_{50} values in syngeneic (AL/N or Balb/c) or in nude mice (cf. B) 1 of Z01 CB 05545-01 IP). Several highly tumorigenic clones ($TD_{50} \leq 10^2$) form rapidly ($\leq$8 weeks) lethal fibrosarcomas similarly to the normal parent clone ($TD_{50} > 10^6$)

possessed very little 55K protein (1/50th to 1/100th of that in SV40 transformed derivative clonal cells). Thus in these mouse fibroblasts, there is no correlation with tumorigenicity in either the syngeneic or in the nude mice.

However, we found other mouse cells (clones), which are considered malignant, which posses the 55K protein in ample amounts. These cells (in addition to RNA virus or methylchorantrane transformed cells investigated by others) include a mouse neuroblastoma clone (neuro 2 a) and an L cell clone (#929). All of these cells, however, do not have closely matched normal counterparts, and have been in culture for a long time.

Clearly our newly isolated and characterized families of cloned cells with careful pedigree are the choice for further controlled biochemical and biologic studies.

Significance to Biomedical Research and the Program of the Institute: Re-expression of certain embryonic antigens in some cancers have been used by others in immunological studies concerning development of certain tumors. Analysis and identification of the embryo protein(s) we have discovered could provide more insight into the mechanism of transformation; also the phase specific expression of this protein could be used at the molecular level to study embryonic differentiation.

Proposed Course of the Project: Specific plans and work underway are given in Z01 CB 05545-01 IP. On a general level those potential avenues will be explored which apply the molecular biology techniques and reagents so well defined in SV40 virology to changes which occur in embryogenesis, and may also relate to control of cell growth rate of both normal and malignant cells. It is hoped to define the cellular gene which is constitutively expressed in normal embryogenesis, the modulation of which occurs in many transformation processes. This is a new type of such cellular gene, different from those (such as the src gene) which have been found to be the cellular homologues of certain RNA tumor viruses: the cellular gene for the 55K protein is not "picked up" in the DNA virus genomes; its expression (at a yet unknown level or levels) is altered (controlled) in many different types of transformations, including the Abelson murine leukemia virus transformed B cells, the methylcholantrane induced mouse tumors, but not the spontaneous transformation of mouse fibroblast. Various potential molecular mechanisms for the modulation of the gene expression will be explored. The nature of the phosphokinase activity in embryo cells and in embryonal carcinomas will be studied by biochemical and immunologic techniques.

Publications:

Mora, P. T., Chandrasekaran, A., and McFarland, V. W.: An embryo protein induced by SV40 virus transformation of mouse cells. Nature 288: 722-724, 1980.

Mora, P. T., and Chandrasekaran, K.: Role of SV40 Induced Antigens in Transformation and Rejection of Malignant Mouse Cells, and the Detection of an Embryo Protein. In Manson, L. and Nowotny, A. (Ed.): Biomembranes. New York, Plenum Publishing Corporation, 1981, in press.

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PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cell Surface Changes in Transformed Mouse Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. W. Luborsky	Chemist	IP	NCI
Other:	K. Chandrasekaran	Visiting Fellow	IP	NCI
	P. T. Mora	Chief, Macromolecular Biology Section	IP	NCI

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.0	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A study of changes on a molecular level which control cell properties, growth and differentiation, and are possibly involved in the transformation to malignancy as well. A cellular protein found bound to SV40 T antigen (TA) in cultures of SV40 transformed murine cells, and later in normal midgestation murine embryos, was thought, by analogy to TA, also to be involved in control reactions pertaining to DNA replication and cell growth and division. Since TA is known to bind to DNA, the behavior on calf thymus DNA-cellulose columns of extracts of cultured mouse cells was analyzed for information concerning the nature and properties of this 55,000 molecular weight (MW) protein (55K protein) in the presence of TA. Results were compared with those obtained from similarly treated extracts of embryonal carcinoma (EC) cells, which contain the 55K protein. Evaluation of the ability of the 55K protein (complex) to associate with DNA should provide a basis for understanding such interactions within the cell. It is hoped to gain a better understanding of the biological activity and function of such proteins and their contribution to the basic control mechanisms of the cell, as well as to develop better methods for their isolation and purification.

Project Description

Objectives: To gain some insight into the biological activity and function of proteins involved in the basic control mechanisms of cell growth and division and the maintenance of the normal phenotype, and to study the interaction and regulation of cellular and viral control proteins; to improve methods for their isolation and study.

Methods Employed: Tissue culture cell cultivation; cell cloning procedures; polyacrylamide gel electrophoresis; column chromatography, including DNA-cellulose chromatography; ultracentrifugation; 2-dimensional thin-layer electrophoresis and chromatography; various immunological assays to detect tumor antigens.

Major Findings: It is known that the tumor antigen (TA) of SV40 binds to SV40 DNA near the origin of replication and plays a role in the control of viral DNA replication and gene expression. It also has been shown to bind to cell DNA, although its binding site(s) have not been shown to be specific on this DNA. It nevertheless is thought to be responsible for malignant conversion and the maintenance of the transformed state by its effect on cell control mechanisms for growth, division and differentiation. Since TA was known to be involved in control mechanisms concerned with viral replication, and presumably with modifying and/or controlling the infected cell process, the discovery of a host protein, somehow derepressed/stimulated by the viral infection, which was specifically associated with the viral TA provided a basis for the expectation that this host 55K protein too was somehow involved in similar control processes in the normal cell. Such a presumption was supported by the detection of this 55K protein in normal, uninfected, midgestation murine embryos. It was felt that the 55K protein represented a contact point in the regulatory pathways of both the normal cell and the virus-cell systems.

One may study the 55K protein by attempting to clone its gene first, as some workers are currently attempting. More direct, and perhaps more easily interpreted, however, is the study we describe here of this 55K protein obtained directly from the cell, and its effects on the cell.

Our recent work has focussed principally on this 55K protein. We had shown that extracts of ^{35}S-methionine labelled SV40 transformed cells of mouse, hamster and rat contained anti-T serum reactive antigens of MW 94,000 and 19,000 (94K and 19K), the SV40 coded large T and small t antigens, respectively, and the host coded 55K protein, and that this 55K protein was to be found both on the surface and in the nucleus of SV40 transformed cells, in association with the 94K protein. Following reports of the presence in mouse embryonal carcinoma (EC) cell lines of a similar 55K MW protein, precipitated by the same hamster anti-T serum used with the SV40 transformed cells, we found this same protein also in midgestation stage murine embryos, not infected or transformed by SV40 (Z01 CB 05545; Z01 CB 05526). The nature and properties of this protein became a focus of major interest to us. We wished first to isolate the 55K protein in relatively pure native form for further study; to compare the 55K protein from the two cell systems for binding capacity to DNA; and to determine if binding was direct, protein to DNA, or if it required mediation of the 94K MW SV40 TA binding also.

For this purpose we chose the well characterized clonal culture line, 215CSC, an SV40 transformed mouse embryo cell, as an easily available source which we found contains a high concentration of the 55K protein. Since this protein had been found to be complexed with the SV40 large TA, known to bind to DNA, we attempted fractionation of an ^{35}S-methionine labelled cell extract on a DNA-cellulose column, using first calf thymus DNA. For a preliminary test, the 94K and 55K bands were eluted from a polyacrylamide gel electrophoresis (PAGE) pattern of the immunoprecipitated 215CSC mouse cell extract, passed through a DNA-cellulose column, and the fractions analyzed. While interpretation of the results was difficult because of the low levels of radioactivity available, a preliminary assessment indicated that the 94K SV40 TA was bound to the DNA and required a higher pH for its elution, while the free 55K cellular protein was not, and passed through the DNA column without binding, the latter being a negative result. It was felt that the 55K protein obtained directly from the cell extract may behave differently since it would not have been subjected to the various denaturing conditions involved in preparation and elution of the gel bands.

Subconfluent cultures of 215CSC cells in exponential growth were labelled with ^{35}S-methionine and the extracted proteins run on a DNA-cellulose column. Two to four times as much label as was bound did not bind to the column. When this unbound fraction was tested on a fresh DNA-cellulose column the ratio of unbound to bound material in it remained high (10-50 fold). Clearly the first column was not overloaded; the non-bound fraction did not contain any significant proportion of material that was capable of binding to DNA but had not during the first pass through the column. Nearly 10X more of the bound material than of the non-bound material was specifically immunoprecipitated by hamster anti-T serum, thus indicating the close correlation between DNA binding capability and anti-T serum reactivity. PAGE analysis of these immunoprecipitates revealed the usual 94K, 55K and 19K proteins present in both fractions, with the 94K and 56K species present in apparently somewhat similar amounts. Relative to the original proportions of radioactivity in both fractions, however, the bound fraction possessed over 6X more of both species than did the non-bound fraction. Moreover the 55K protein was present in both bound and non-bound fractions, a result contrary to the preliminary indication obtained from the 94K and 56K bands eluted from the gels.

The eluates were further analyzed by sucrose density gradient centrifugation, to determine the size distribution of the protein complexes as originally obtained from the cells, under less denaturing conditions. Generally, sedimenting peaks were seen at molecular weight positions of about $(17\text{-}22)\times10^3$, 45×10^3 and $(86\text{-}107)\times10^3$ daltons. Thus, in these solutions the size of various proteins present seems to correlate as uncomplexed proteins with the three major anti-T serum reactive antigens mentioned above. Work is continuing now to explore further the nature, amounts and properties of the 55K protein obtained from the DNA-cellulose column and the density gradient sedimentation procedures.

Another aspect of this study of the nature of the 55K protein derives from its presence in embryo cells not infected with SV40. An embryonal carcinoma cell, F9, was studied in much the same fashion as indicated above. Extracts of ^{35}S-methionine labelled EC cells were treated in this fashion and fractions which eluted from the DNA-cellulose column were analyzed. A large non-bound fraction

eluted first, following by 2 well separated bound peaks, the first eluted by the higher pH buffer (pH 8), the second, by this buffer containing 0.7M salt. The non-bound peak,and the loosely bound peak eluted at higher pH,both appear similar to those obtained from the 215CSC cell extracts, while the more tightly bound high salt peak was not seen before. Although the three peaks eluted from the DNA constituted 89%, 6% and 3%, respectively of the effluent, after dialysis, concentration and redissolving in buffer suitable for immunoprecipitation, 30%, 6% and 1.3%, respectively, remained. Following immunoprecipitation with hamster anti-T serum, samples are being analyzed by PAGE and fluorography. Interpretation of the nature and composition of these peaks awaits the results of this analysis.

Significance to Biomedical Research and the Program of the Institute: It is important to try to understand better the nature and properties of the cell components which exert important influences upon cell growth characteristics and the ability of certain cells either to replicate normally, to form tumors or be rejected by the host, or to differentiate in a normal orderly fashion. The 55K protein apparently provides a unique activity for study, representing as it does a contact point in the regulatory pathways of two systems, the normal cell and the virus-cell systems. As already pointed out, we have found this protein together with the SV40 TA, on the surface of SV40 transformed murine cells, as well as in their nuclei. The presence of such a regulatory protein on the surface may provide a link to mechanisms of surface control of cell growth and division. Many cell systems have recently been shown to possess this 55K protein, including cells of both virus infected or transformed or tumor origin, and uninfected normal cells, including midgestation murine embryos which we have studied (Z01 CB 05545; Z01 CB 05526). Such widespread occurrence underscores the apparent biological importance of this 55K protein (complex). It is hoped that evaluation of its ability to associate with DNA will provide a model for understanding better such interactions within the cell. These interactions may be the basis of the effect of some proteins in regulating differential cell growth and differentiation. Study of its behavior in these systems should contribute to a better understanding of basic cell processes at the molecular level.

Proposed Course of the Project: The nature of the binding of the 55K protein to DNA will be investigated, first to determine whether it can bind directly to DNA or requires a mediator, in the form of another protein such as the SV40 TA. Attempts will be made to fractionate the SV40 anti-T reactive proteins from various sources to obtain each free of the others and not denatured by high concentration of detergent, urea or other denaturing solvent, to compare the 55K proteins from each source. Particular attention will be focussed on possible differences in properties and/or function of the 55K protein obtained from virus transformed cells or from normal embryos or F9 EC cells. We will attempt to better understand the relevance of the various changes in cell biochemistry and immunochemistry, and the presence and function of the 55K protein, to spontaneous and to viral induced transformation, as well as to normal differentiation of cells.

Publications:

Chandrasekaran, K., Winterbourne, D. J., Luborsky, S. W., and Mora, P. T.: Surface proteins of simian virus 40 transformed cells. Int. J. Cancer 27: 397-407, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05078 04 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to Influenza-Infected Autologous Lymphocytes in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. E. Biddison	Cancer Expert	I	NCI
	J. S. Shaw	Senior Investigator	MET	NCI
Other:	G. M. Shearer	Senior Investigator	I	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro stimulation of influenza virus-immune cytotoxic effector cells in human peripheral blood leukocyte (PBL) populations; and (b) measurement of effector cell activity on virus-infected cryopreserved human PBL target cells was investigated. Effector cells generated in vitro are influenza type-specific and are HLA-A and HLA-B restricted. The cytotoxic response appears to be controlled by HLA-linked immune response (Ir) genes since: (a) cytotoxic activity is predominantly associated with antigens of only one haplotype; and (b) effector cells from certain donors recognize virus in association with some but not all self HLA-A and -B antigens. This Ir gene control is HLAlinked and antigen-specific, since preferential recognition among HLA-identical siblings is different for influenza A and B viruses. Comparative studies of HLA-A positive and of HLA-A3 positive donors for these CTL responses indicate that greater heterogeneity of these HLA antigens can be demonstrated by CTL than by current serology. The CTL response to influenza virus was found to depend on interacting populations of helper and CTL precursor T cells.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The primary objectives of this laboratory are to investigate the function of T lymphocytes, the role of self recognition, and the effects of major histocompatibility genes on the murine and human immune systems. These studies are being pursued using mouse and human leukocytes which are sensitized to autologous cells either modified with chemical agents (e.g. the trinitrophenyl group) or infected with viruses (e.g. influenza, measles). The objectives of this project are to: (a) define the components of the human major histocompatibility complex (HLA) which are recognized in association with viral antigens by cytotoxic T cells; (b) analyze the virus specificity of human influenza immune cytotoxic T cells; (c) determine if there is any "Ir-like" genetic control of human T cell response potential to influenza viruses; (d) characterize the human cell populations required to generate virus-immune cytotoxic T cells; and (e) to determine whether different sites or epitopes on the HLA molecule can be identified which serve as self recognition structures for recognition of virus.

Methods Employed: Human PBL were obtained from normal adult volunteers, separated on a Ficoll-Hypaque gradient, and sensitized in vitro to live influenza viruses. Cytotoxic activity was measured by ^{51}Cr release from cryopreserved peripheral blood cells which were thawed, stimulated with PHA, and infected with influenza virus. The OKT series of monoclonal antibodies was used in association with the FACS II and cell separation by monolayers to positively and negatively select subpopulations of human T cells required for CTL responses to influenza virus.

Major Findings: The human cytotoxic T cell response in vitro to type A influenza viruses is predominantly directed against cross-reactive determinants on cells infected with serologically distinct type A influenza viruses. Reciprocal exclusion of cytotoxicity was observed between effectors sensitized to type A and B influenza viruses. Human influenza virus-immune cytotoxic T cells recognize viral determinants predominantly (>90%) in conjunction with self antigens that are encoded by genes closely linked to HLA in family studies and that are highly associated with the HLA-A and -B loci in population studies. In a family study, siblings consistently generated cytotoxic activity against influenza predominantly in association with antigens coded by genes of only one of their HLA haplotypes. Such haplotype preferences were consistent among HLA-identical siblings, indicating that the specificity of the T cell response to influenza virus in association with HLA-A and -B antigens is controlled by genes linked to HLA. Virus-immune effectors from certain donors recognize virus in conjunction with some, but not all, of their self HLA-A and -B antigens. Among donors who who share a given HLA antigen (such as A2 or B7), there are differences in the ability of their virus-immune T cells to recognize the shared antigen. Virus-infected target cells from HLA-A2 or B7 "nonresponder" donors could be lysed by virus-immune T cells obtained from other donors who shared only the HLA-A2 or -B7 antigen with these target cells. These observations suggest that the absence of cytotoxic T cell responses by some donors to influenza virus in conjunction with particular self HLA-A and -B antigens is not due to control by the structural genes which code for these HLA antigens, but rather may result from control of regulatory genes which act

at the level of the responder and/or stimulator cell. An individual with a variant HLA-A2 molecule has been detected by influenza-specific and allogeneic cytotoxic responses. This variant is not detectable by serotyping, but has been verified by HLA peptide analysis. Similar observations have been made in which variant HLA-A3 donors have been identified by influenza-specific CTL. Cell fractionation studies using the OKT series of monoclonal reagents indicate that influenza-specific CTL precursors are $OKT3^+$, $OKT4^-$ and $OKT8^+$, whereas helper T cells are either $OKT4^+$ or $OKT4^-$.

Significance to Biomedical Research and the Program of the Institute: This project is of fundamental immunological importance, since: (a) it provides one of the first known examples of cell-mediated immunity which involves self-recognition by human lymphocytes, and (b) it involves a virus which is of significance in human disease. Furthermore, this system can be used to test a number of major histocompatibility-linked immune phenomena in man which are known to occur in mice (e.g., MHC-restriction, haplotype preference, Ir gene control, and cell-to-cell interactions).

Proposed Course of Project: Further studies will be performed using lymphocyte populations from unrelated HLA matched and unmatched individuals, and from members of families including those known to be recombinants within the HLA complex in order to better define the components of the HLA complex which are recognized in association with viral antigens. Studies will be performed to examine antigen specificity of HLA-linked genetic control of virus-immune T cell specificity. Preliminary results suggest that there are differences in the HLA haplotype preferences observed between T cells sensitized to A/HK and B/HK influenza viruses. In population studies, there are differences between A/HK and B/HK-immune T cells in recognition by T cells may help to determine whether different repertoires for foreign antigens exist for T cells which recognize different self determinants. We plan to determine if differences in HLA recognition by virus-immune T cells are due to differences in the responder and/or stimulator cell functions. In order to use responder and stimulator peripheral blood leukocytes from different individuals for generation of virus-immune cytotoxic T cells, it is first necessary to remove any responder cells capable of generating an alloimmune response against the stimulator cells. To remove this alloreactive potential, we are developing a negative selection technique based on specific adherence of T-cells to monolayers of cells expressing alloantigens. We plan to investigate whether influenza virus can be recognized in association with different HLA-coded self determinants by attempting to block the cytotoxic reaction with monoclonal antibodies directed against distinct parts of HLA molecules. Studies will be continued using the OKT series of antibodies to identify other populations of T cells. Biochemical studies of the HLA-A3 variant donors will be made to determine whether there is molecular evidence for these variants.

Publications

Biddison, W. E., Krangel, M. S., Strominger, J. L., Ward, F. E., Shearer, G. M. and Shaw, S.: Virus-immune cytotoxic T cells recognize structural differences between serologically indistinguishable HLA-A2 molecules. Human Immunol. 3: 225-232, 1980.

Biddison, W. E., Payne, S. M., Shearer, G. M., and Shaw, S.: Human cytotoxic T cell responses to trinitrophenyl hapten and influenza virus: Diversity of restriction antigens and specificity of HLA-linked genetic regulation. J. Exp. Med. 3: 225-232, 1980.

Biddison, W. E., Ward, F. E., Shearer, G. M. and Shaw, S.: The self determinants recognized by human virus-immune T cells can be distinguished from the serologically defined HLA antigens. J. Immunol. 124: 548-552, 1980.

Biddison, W. E., Sharrow, S. O., and Shearer, G. M.: T cell subpopulations required for the human cytotoxic T lymphocyte response to influenza virus: evidence for help. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05081-04 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Ontogeny of Immune Responsiveness in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Nadler	Investigator	I NCI
Other:	R. J. Hodes	Chief, Immunotherapy Section	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ability of mammalian species to respond to in vivo challenges with complex antigens develops in a time dependent manner during embryonic and neonatal life. Functional or structural immaturity of B cells, T cells, and accessory cells in fetal or neonatal animals as well as active, non-specific suppression of humoral and cellular immune responses by neonatal immunocytes has been reported. Utilizing two systems, 1) the in vitro primary antibody response to soluble hapten-protein conjugates, and 2) the one way mixed lymphocyte response, it has been shown that thymic T cells and both splenic T and non-T cells are capable of actively suppressing immune response by cells from adult animals. The splenic adherent cell population (SAC) isolated from neonates was also shown to be an inefficient antigen presenting accessory cell. These neonatal SAC were also inefficient stimulators of MLR across both H-2 and Mls differences. Fluorescent microscopic studies revealed that fewer neonatal SAC were Ia positive than adult SAC. Moreover the development of functional accessory cell activity with age paralleled the expression of Ia antigens by SAC.

Project Description

Objectives: Investigation is under way concerning the maturation of the immune response in mice to soluble hapten-protein conjugates. Inability to respond to various antigens has been noted in many species during embryonic development and in the neonatal period. Spleen cells from 7 to 10 day old mice are unable to respond in vitro to soluble TNP-KLH as measured by a plaque forming cell assay of primary IgM antibody response. The mechanism of this neonatal unresponsiveness is the object of this project.

Methods Employed: The major experimental systems employed include in vitro primary antibody responses as measured by a plaque forming cell response, the one way mixed lymphocyte response and cell surface immunofluorescent labelling. Cell separation procedures include nylon non-adherence to obtain populations enriched in T cells; RαMB and complement treatment and G10 Sephadex passage to obtain populations enriched in B cells, G10 Sephadex passage to obtain macrophage or accessory cell depleted populations and glass adherence for macrophage or adherent cell populations.

Major Findings: The ability of spleen cells to respond to soluble TNP-KLH appears approximately 2-3 weeks after birth in BALB/c mice with inconsistent responses prior to this period. Responses appear maximal between 2-7 months of age. The nature of neonatal unresponsiveness in vivo and in vitro could result from active suppression in neonatal spleen cells and/or from defects in the functional capacity of one or more neonatal populations. In the present study, unfractionated neonatal spleen cells consistently suppressed antibody responses by adult spleen cells. Attempts to isolate a population containing the suppressor cell(s) led to the finding that both "T-enriched" (nylon-non-adherent) and "non-T" (RαMB+C treated and/or G10 passed, RαMB+C treated) populations could mediate this suppression. Treatment with T cell specific reagents (RαMB, αθ ascites and an αθ hybridoma) failed to abrogate the suppressor activity in neonatal spleens. Treatment of neonatal spleen with αIa antisera and C also had no effect on this active suppression. Studies aimed at determining the immune competence of subpopulations of neonatal spleen (e.g. T cells, B cells, and macrophages) were complicated by the presence of active suppressor cells in both the T and non-T cell subpopulation. The function of accessory cells could be investigated because of the relative radioresistance of accessory cell function and the relative radiosensitivity of the suppressor cell activity of neonatal spleen cells. The ability of neonatal and adult adherent spleen cells to function as accessory/antigen presenting cells was assessed for in vitro primary antibody responses to TNP-KLH. Neonatal splenic adherent cells (SAC) were shown to be less efficient antigen presenting accessory cells than adult SAC and this ability was shown to mature in a time dependent manner. It was demonstrated that fewer latex-phagocytic cells in the neonatal adherent spleen cell populations expressed cell surface Ia antigens than adult splenic latex-phagocytic cells. The expression of Ia antigens on latex-phagocytic cells progresses from the neonatal period into adult life and reaches plateau values approximately 6 weeks after birth. The appearance of an increasing proportion of Ia positive, latex-phagocytic cells in the SAC subpopulation was shown to

correlate highly with the ability of this population to function as antigen presenting accessory cells for in vitro responses to TNP-KLH. The observation that cells within the SAC population are the major stimulators of the murine mixed lymphocyte response (see Project # Z01-CB-05069-03 I) led us to investigate the ontogeny of cellular stimulatory ability in the MLR. As was observed for accessory and antigen presenting function, neonatal SAC were less potent stimulators of T cell proliferation than adult SAC when the stimulator and responder strains differed either at the Mls locus, the entire murine major histocompatibility complex (H-2) or subregions within H-2. The development of this ability to stimulate mixed lymphocyte responses across I region-encoded determinant differences paralleled the ontogenetic rate of expression of these antigens on SAC as examined by immunofluorescent techniques. The ability of neonatal and adult SAC to stimulate either allogeneic CML or "TNP-modified self" CML was comparable and fluorescent investigation of expression on K region encoded determinants on these cells revealed that the majority of both newborn and adult SAC bore these determinants. It was also confirmed that sIgM positive splenic (B) cells are virtually all Ia antigen positive by the end of the first postnatal week.

Significance to Biomedical Research and the Program of the Institute: It is known that immune responsiveness in neonatal and senescent animals is deficient relative to adult animals of that species. Information concerning the mechanisms of neonatal immune deficiency is incomplete and investigations in this area will be applicable to the process of differentiation and to possible means of modulation of responsiveness. It is also known that the incidence of malignant neoplasms is greatest at the extremes of life and it has been postulated that defects in "immunologic surveillance" may play a role in this occurrence. Studies of neonatal cellular competence and the mechanism of action of neonatal suppressor cells may shed light on these postulated defects. This work demonstrates that neonatal immunoincompetence is the result of a complex interplay of suppressor cell influence and immaturity of one or more of the cell population required for responsiveness. Evidence has been accumulated that the ontogenetic rate of expression of Ia antigens differs on different populations of cells involved in immune responses and that the expression of Ia antigen on cells within the SAC population is fundamental for their cooperation in antibody responses.

Proposed Course of Project: Continuing studies will pursue the cellular causes of the inability of neonatal animals to respond to soluble antigens. Specifically, the ability of neonatal SAC to provide accessory cell function for responses to T independent, macrophage dependent antigens like TNP-Ficoll will be investigated. In addition, means of inducing expression of Ia antigens on cells within the SAC population will be investigated.

Publications

Nadler, P. I., Klingenstein, R. J., and Hodes, R. J.: Ontogeny of neonatal accessory cells. Ia antigen expression and function in in vitro primary antibody responses. J. Immunol. 125: 914-920, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05083-03 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Genome Organization of Murine Major Histocompatibility Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. Singer	Senior Staff Fellow	I NCI
Other:	D. H. Sachs	Chief, Transplantation Biology Section	I NCI
	S. Rudikoff	Senior Investigator	LCB NCI
	L. Abelson	Biologist	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	5.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study was initiated to determine the DNA sequence organization of the genes encoding the major histocompatibility complex and the mechanisms controlling the expression of these genes.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: This laboratory has undertaken to study the molecular biology of the major histocompatibility locus in the mouse and the miniature swine. In both species, it is known that the structural products regulated by this region of the genome are highly polymorphic and responsible for both transplantation rejection and regulation of immune responses. Biochemical studies have demonstrated that the MHC antigens of mouse and miniature swine are structurally homologous.

Genetic studies have shown that the genes involved in regulating the expression of the transplantation antigens, as well as those involved in regulating the immune response are all linked on a single chromosome. However, the organization of these genes varies between the two species. Therefore, this region is of considerable interest in studying the regulation of a coordinated set of functions. The objects of the studies are to characterize the genome organizations and regulation of these families of genes in each of the two species and to analyze the evolutionary relationship between them.

Methods Employed: Two separate approaches are being taken to attempt to purify the genes of these MHC loci. In the first, the mRNA species encoding MHC products are being purified from the spleens of each of the species under study. The purification is monitored in two ways: by the ability of various mRNA fractions to direct the cell free synthesis of the structural products and by their ability to hybridize to a heterologous human MHC probe. The purified mRNA species will then serve as templates in the synthesis of double stranded cDNA which will be propagated in recombinant bacterial plasmids.

The alternative approach to studying the genomic organization of the MHC locus involves the direct isolation of the individual genes encoding the various structural products. Total mouse DNA, isolated from the livers of inbred strains of mice and miniature swine, is enzymatically fragmented, and each fragment is inserted into a viral vector. These recombinant DNA libraries are then screened using a heterologous human MHC cDNA probe. Isolated genomic clones are characterized either by direct DNA sequence analysis or by the ability of these genes to direct the synthesis of MHC products. The isolation of such genomic MHC genes will allow an analysis of the organization of a single constitutively expressed gene, as well as its relationship to other members of a multigene family.

Major Findings: Through analysis of splenic RNA by molecular hybridization, it has been determined that the RNA species encoding transplantation antigens are all approximately 16S in size. In contrast, in the pig there appear to be multiple RNA species homologous to MHC genes. The major species is approximately 14S, somewhat smaller than the murine MHC RNA. The remaining species are all larger. The relationships between these different molecules remains to be determined.

Studies on the in vitro synthesis of MHC products have yielded new insights into the biogenesis of MHC products. PolyA$^+$-RNA was isolated from mouse spleen, translated in a variety of in vitro translational systems, and subsequently identified by selective immunoprecipitation. Translation of polyA$^+$-RNA in a cell-free reticulocyte lysate system results in the synthesis of precursors of MHC products which display the gross structural features of the MHC antigens, but not those features unique to the mature, membrane-bound MHC antigens. The generation of mature MHC products in vitro requires the presence of a membrane fraction during synthesis. This was demonstrated by translating polyA$^+$-RNA in either frog oocytes or in the reticulocyte lysate supplemented with dog pancreatic microsomes. In these cases, the observed product has acquired the unique structural features of the mature MHC antigens. Cell free translation of size fractionated mouse RNA species has confirmed that the RNA species encoding murine transplantation antigens have sedimentation coefficients of approximately 16S; further, RNA species encoding Ia antigens have been determined to be 12S.

Screening of both mouse and pig recombinant DNA libraries with the human MHC cDNA probe has identified a number of genomic fragments which contain MHC genes. These recombinant phages are presently being characterized to determine which members of each multigene family are represented in the recombinant DNA.

Significance to Biomedical Research and the Program of the Institute: Genetic studies in a number of mammals, including man, mouse and guinea pig, have demonstrated the existence of immune response genes which control cellular interactions leading to both humoral and cellular immunity. The inability of an animal to respond to a given antigen or to reject foreign tissue probably represents a genetic defect. Despite the clear importance of the major histocompatibility locus in the immune response, nothing is known at the molecular level about the content, genetic organization or regulation of expression of this multigene family. An understanding of the molecular basis of the MHC may afford the possibility of treating various immunodeficiency diseases by appropriate genetic manipulations.

Proposed Course of Project: The analysis of the organization of the genes encoding the MHC will proceed along two lines: (1) Purification of the mRNA species encoding the MHC products in both mouse and pig. Once purified, these mRNA molecules will be used as templates to synthesize double-strand cDNA, which will be propagated in recombinant plasmids. It will then be possible to analyze the various MHC RNA species found in the pig to ascertain their relationships to each other and to compare these expressed sequences with those found in the mouse.

(2) Characterization of isolated MHC genomic clones. The genomic clones already isolated will be analyzed to determine which MHC products they encode. Once this has been established, it will be possible to determine the evolutionary relationships between the pig and mouse MHC gene families. It should also be possible to begin to examine the regulation of expression of these genes.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05084-03 I

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Antibody Dependent Cellular Cytotoxicity.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Wunderlich	Senior Investigator	I NCI
OTHER:	J. Connolly	Microbiologist	I NCI
	D. Segal	Senior Investigator	I NCI
	J. Titus	Chemist	I NCI
	S. Dower	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.2	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human peripheral blood lymphocytes with augmented ADCC activity resulting from 2-day in vitro stimulation by Concanavalin A have been quantitatively assessed by flow microfluorometry for IgG Fc receptor density, affinity and distribution profiles. Analysis of 100,000 cell aliquots of Con A stimulated and control, non-stimulated cells from each of four donors revealed that 4-25% of cells bore IgG Fc receptors (FcR). Analysis of the FcR positive cells from each of the 4 donors showed that the average receptor density for a donor ranged from 65,000 to 72,000 receptors per cell, assuming that each subunit of Fc receptor ligand (dimeric anti-DNP) bound to an Fc receptor. The average binding constant per donor ranged from 1.8 to 2.6×10^7 (M^{-1}). Con A stimulation did not have a consistent effect on either of these parameters, and the changes that did occur represented less than 10% of the values for control cells. All fluorescence distribution profiles of cells with fluorescence labeled Fc receptor were unimodal and nearly overlapping for Con A stimulated and control cells.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: This project has been directed at understanding the cellular nature and mechanism of antibody dependent cell-mediated cytotoxicity (ADCC) mediated by activated human lymphocytes.

Methods Employed: Heparinized peripheral blood cells are enriched for leukocytes by sedimentation in Plasmagel and are depleted of monocytes and red blood cells by carbonyl iron ingestion followed by Ficoll-Hypaque cell separation. The cells are cultured for 1-2 days in medium with 20% allogeneic serum, during which time they are stimulated with the mitogen Concanavalin A.

Different doses of stimulated or control lymphocytes are then coincubated as effector cells for 4 hrs. with ^{51}Cr-labelled target cells, pretreated with antiserum from a multiply transfused aplastic donor. Usually a human B lymphoblastoid cell line has been used as a source of target cells. Presensitized with IgG antibodies, these target cells are highly sensitive to ADCC and provide a linear assay for effector cell activity.

The affinity and density of IgG Fc receptors on lymphocyte surfaces are determined using covalently cross linked dimers of rabbit anti-DNP antibodies. Lymphocytes treated first with different doses of dimer and then with an excess of fluorescein-conjugated affinity purified Fab^2 goat rabbit IgG are analyzed for cell fluorescence using a FACS II instrument. Dr. Segal previously demonstrated that: (1) dimers bound to cell surface Fc receptors are relatively resistent to washing procedures, (2) dimers bound to cell surface Fc receptors remain cell surface associated after washing procedures, (3) the procedures for labelling IgG dimers bound to Fc receptors with fluorescent anti-IgG antibody do not affect the amount of bound dimer, and (4) fluorescent emission is a linear function of the number of IgG dimers bound per cell.

Major Findings: Previously, we demonstrated that stimulation of human peripheral blood lymphocytes in vitro with Con A enhances effector cell activity for ADCC. Analysis of receptors for antibody (FcR) on the surface of effector cells showed no change in binding profiles (affinity) or density. Availability of more specific reagents has now permitted direct quantitation of these parameters. Analysis of 100,000-cell aliquots of Con A stimulated and control, non-stimulated cells from each of 4 donors revealed that 4-25% of cells bore IgG-Fc receptors. Analysis of the FcR positive cells from each of the 4 donors showed that the average receptor density for a donor ranged from 65,000 to 72,000 receptors/cell, assuming that each of subunit of dimeric anti-DNP bound to an Fc receptor. The average binding constant was $1.8\text{-}6.9 \times 10^7$ (M^{-1}). Con A stimulation did not have a consistent effect on either of these parameters and what changes did occur represented less than 10% of the values for control cells. All of the fluorescence distribution profiles of cells whose FcR were labelled with different concentrations of antibody dimer were unimodal, and Scatchard plots of the average antibody binding level as a function of antibody concentration were linear--both findings showing no evidence for heterogeneity in binding constants. Dimer binding profiles of Con A stimulated and control cells nearly overlapped when

compared for each donor at the different concentrations of antibody dimer. An important point of this analysis is that it was carried out at 0° C. At higher temperatures, increased membrane fluidity induced by Con A stimulation might increase the effective FcR affinity for antibody.

Significance to Biomedical Research and the Program of the Institute: Antibody dependent cellular cytotoxicity provides a mechanism whereby normal lymphoid cells can be utilized for target cell destruction. Evidence has accrued from a variety of laboratories that the mechanism may operate in human rejection of certain parasites, fungi, viruses, tumor-cells and allografts. ADCC activity is dependent on and probably proportional to the Fc receptor density on killer cells and the affinity of these receptors for immunoglobulin. Accurate quantitation of these parameters is thus important not only for comparing different types of effector cells (e.g. activated vs. non-activated) but also for gauging FcR site availability to antibody coated target cells.

Proposed Course of Project: A major technical advance is needed for significant future progress on this project: a means for purifying the sub-population of lymphocytes which destroy antibody treated target cells (K cells). Rapid advances in generation and characterization of hybridoma monoclonal antibodies and lectins which react with differentiation determinants on human lymphocytes are now occurring in other laboratories and it is hoped that these reagents will provide the means of purifying K cells. Thus, future work on this project will be delayed until the specificity patterns of these reagents are more clear. Publications for this project will be incorporated under other projects by this investigator and this project will be terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05085-03 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Role of Cell Interactions in the Development of Syngeneic Tumor Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Fujiwara	Visiting Associate	I	NCI
Other:	G. M. Shearer	Senior Investigator	I	NCI
	T. Tsuchida	Visiting Fellow	I	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This work was initiated to study the role of cell interactions in the development of syngeneic tumor immunity. It has been shown that the degree of specific anti-tumor immunity developed both in vivo and in vitro in response to TNP- conjugated tumor cells can be increased by the addition to TNP-reactive helper T cells. The cytotoxic cell in vitro has been shown to be a T-cell. The nature of the lymphocytes involved in in vivo killing is under investigation.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The aim of this project is to study the role of cell interactions in syngeneic tumor immunity. Previously, a system was explored in which animals were immunized with syngeneic tumor cells modified with additional determinants. Supplementation of helper (amplifier) T-cells specific for those determinants amplified the generation of killer T cells against native tumor antigens. Specifically, helper T-cell activity against 2,4,6-trinitrophenyl (TNP) hapten was elicited by immunizing mice with TNP-conjugated isologous mouse gamma globulin (MGG). Addition of TNP-helper T cells during the immunization of C3H/He mice with TNP-conjugated syngeneic tumor cells led to a significant enhancement of killer cell activity against the native, unmodified tumor.

These preliminary results were observed after several immunizations with TNP-conjugated tumor cells. The requirements for producing optimal augmentation of in vitro cytotoxic effector cell activity and in vivo protective immunity, as well as the nature and specificity of in vitro and in vivo effector mechanisms augmented by the above cell-cell interaction system are the subject of the current project.

Methods Employed: TNP-reactive helper T-cells were induced by intraperitoneal (i.p.) inoculation with 500 ug TNP-D-GL in saline, followed by i.p. injection of 100 ug of syngeneic TNP-MGG in complete Freund's adjuvant (CFA) 3 days later. Six weeks after the immunization with TNP-MGG, animals were inoculated i.p. with 1.5×10^7 TNP-conjugated LSTRA tumor cells at 10- to 14-day intervals. Two weeks after the final immunization with TNP-LSTRA, spleen cells were sensitized to in vitro and cytotoxic effector cells were measured by ^{51}Cr release assay in vitro and in vivo tumor-neutralization test.

Major Findings: The study indicates that the addition of TNP-reactive helper T-cells to the BALB/c-LSTRA syngeneic tumor system at the time of immunization with TNP-conjugated LSTRA tumor cells results in the accelerated development as well as the amplified generation of tumor-specific cytotoxic effector T-cells in vitro and tumor-neutralizing T-cell activities in vivo. We have also demonstrated the absolute requirement of the TNP-reactive amplifier T-cell system for the induction of a powerful in vivo immunity as observed in the Winn assay.

Significance to Biomedical Research and the Program of the Institute: The role of helper cells in immune reactions may be clarified and possible applications to the immunotherapy of tumors made possible.

Proposed Course of Project: Additional research should be performed to (1) establish a simpler and more effective condition under which strong amplifier T-cells can be induced and (2) determine the nature of the amplifier T-cells and cells which are responsible for in vivo immunity. Helper cell activity will be analyzed as a possible approach for augmenting cell-mediated immunity to tumor antigens. Helper activity will be raised by immunization

against TNP and other haptens. Such helper cells will be tested for their potential to effect more efficient immunity to tumor antigens. In these studies the B16 mouse melanoma line as well as radiation-induced lymphoid tumors will be studied both in the parental strain of origin and in F_1 hybrid strains to determine whether F_1 resistance to the tumor can be detected and manipulated by helper cells.

Publications

Fujiwara, H., and Shearer, G. M.: Suppressive effect of X-irradiated tumor cell presensitization of the induction of syngeneic tumor immunity. II. Opposite effects of intravenous administration of TNP-conjugated tumor cells on the development of anti-tumor and anti-TNP-self cytotoxic effector cells. Cellular Immunol. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05086-03 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immune Response Gene Regulation of the Immune Response In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Hodes	Chief, Immunotherapy Section	I NCI
	P. I. Nadler	Investigator	I NCI
Other:	A. Singer	Senior Investigator	I NCI
	G. Miller	Investigator	I NCI
	D. H. Sachs	Chief, Transplantation Biology Section	I NCI
	Y. Asano	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The cellular expression of immune response (Ir) gene function was studied in an in vitro system of primary antibody responses to the TNP conjugates of (T,G)-A--L and (H,G)-A--L. These responses require the participation of T cells and accessory cells as well as B cells, and are under the control of genes mapped to the K or I-A subregions of the H-2 complex. It was demonstrated that the function of accessory cells in responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L is under the control of genes which also map to K or I-A. In contrast, both B cells and T cells from nonresponder H-2^a strains to TNP-(T,G)-A--L are competent in supporting primary antibody responses to this antigen, and therefore do not appear to express the Ir gene defect present in these strains. Subsequently, in vitro augmented primary responses to TNP-nuclease (TNP-NASE) have been established and documented to be under the control of H-2 linked Ir gene(s). For these responses as well, accessory cell function was shown to be under Ir gene control. Through the use of intra-H-2 recombinant strains, the Ir gene(s) controlling responsiveness to TNP-NASE were shown to map to I-B.

Project Description

Objectives: The major objective of this project is to investigate the mechanism of genetic regulation of antibody responses. Initial studies identified the cellular level of Ir gene expression for the in vitro responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L. Currently ongoing studies are directed at characterizing the Ir gene control of responses to TNP-NASE, including the possible function of gene complementation in this control.

Methods Employed: The methods employed have been described in detail. See project No. Z01 CB 05064-04 I.

Major Findings: Background work has demonstrated that primary or augmented primary in vitro antibody responses could be generated to a number of soluble TNP conjugates of protein or polypeptide antigens. These responses are both T cell-dependent and accessory cell-dependent. The in vitro primary IgM responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L are under strict H-2 linked gene control by genes mapping to the K or I-A subregions. Accessory cell function is under H-2 linked Ir gene control, mapping to K or I-A, while neither B cells nor T cells of the H-2^a haplotype express detectable Ir gene defects for the response to TNP-(T,G)-A--L.

An in vitro system was established in which TNP-specific responses to TNP-conjugated Staphylococcal nuclease (TNP-NASE) were generated by spleen cells from NASE-primed mice. These responses were T-cell and accessory cell-dependent, and under H-2-linked Ir gene control, with strains of the H-2^a haplotype being responders and H-2^b strains nonresponders. Ir gene control mapped to I-B and was not explained by complementing genes in I-A and I-E/C. Cell fractionation experiments have shown that accessory cell function is under Ir gene control for the response to TNP-NASE. Experiments carried out with a hybridoma anti I-A^k reagent have demonstrated that this reagent is capable of inhibiting the response of (H-2^a x H-2^b)F_1 spleen cells to TNP-NASE. These findings suggest that a gene (or genes) in I-A, as well as genes in I-B may regulate the response to TNP-NASE.

Significance to Biomedical Research and the Program of the Institute: Genetic control of immune responses has been demonstrated in widely studied systems, including those responses to biologically "natural" antigens including allergens, viral determinants, and tumor antigens. In order to understand the mechanism of differentially reactivity and susceptibility to these natural stimuli, the mechanism of Ir gene regulation of responses to defined stimuli may provide informative insights.

Proposed Course of Project: Conventional and monoclonal anti-Ia antibodies will be used to probe for the I region products which function in Ir gene expression.

Publications

Dickler, H. B., Cowing, C., Ahmann, G. B., Hathcock, K. S., Sachs, D. H., Hodes, R. J. and Singer A.: Expression of Ir genes and Ia antigens by adherent accessory cells required for antigen-specific antibody forming cell responses. In van Furth R. (Ed.): Mononuclear Phagocytes. Functional Aspects, Part II. The Hague, Martinus Nijhoff B.V.-, 1980, pp. 1909-1922.

Nadler, P. I., Miller, G. P., Sachs, D. H., and Hodes, R. J.: Ir gene control of in vitro antibody responses to TNP-nucelase. J. Immunol. in press.

Singer, A., Hathcock, K. S., and Hodes, R. J.: Self-recognition in allogeneic chimeras. A radiation-resistant host element dictates the self specificity and immune response gene phenotype of T-helper cells. J. Exp. Med., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05087-03 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

T Cell Recognition in the Mixed Lymphocyte Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Hodes	Chief, Immunotherapy Section	I NCI
	R. Gress	Clinical Associate	I NCI
	P. Nadler	Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1	.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ability of subpopulations of murine spleen cells to stimulate a mixed lymphocyte response (MLR) was studied. It was found that T cells and B cells were poor stimulators of an MLR across H-2 or Mls differences, while non-T, radiation resistant Ia positive splenic adherent cell (SAC) were 20-50 times more efficient as stimulators of these MLR's than unseparated spleen cells. These results suggest that Ia^+SAC may be the predominant stimulating cells in spleen cell populations, and the preferential target for T cell recognition in cell interaction events. It has further been demonstrated that T cells responding to Mls determinants recognize such determinants in the context of "self" H-2 determinants. Studies employing K region mutants have investiated the influence of chimeric maturation environment upon the alloreactive T cell repertoire to mutant determinants, and suggest that this alloreactive repertoire is in fact influenced by the environment in which T cells mature.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Since the MLR is a model for T cell recognition of cell surface determinants, the objective of these studies is to characterize T cell recognition of allogeneic determinants.

Methods Employed: Purified T cells (nylon non-adherent spleen cells) purified B cells (G10 Sephadex passed and RAMB + C treated) and splenic adherent cells (glass adherent, non-T radiation resistant spleen cells) (SAC) were prepared and compared for their ability to stimulate a proliferative response by allogenic whole spleen cells. Stimulator SAC populations are further evaluated by 1) treatment with anti-Ia reagents and C, 2) addition of monoclonal anti-Ia and anti-H-2 antibodies directly to the culture, and 3) fractionation of SAC on the basis of their ability to phagocytise latex particles. In vitro generation of cytotoxic T lymphocytes (CTL) was carried out by MLR cultures.

Major Findings: A non-T radiation resistant spleen adherent cell population (SAC) was up to 20-50 times more efficient in stimulating MLR on a per cell basis than an unseparated spleen cell population; and these SAC express Ia determinants encoded by genes in I-A and I-E/C. These findings were observed both for MLR to H-2-differences and for MLR to Mls stimulating determinants.

Mls encoded determinants appear to be unique in that they are the only non-MHC determinants capable of stimulating primary MLR. Studies were undertaken to determine whether responding T cells recognize Mls product alone, or recognize Mls in the context of H-2. Limiting dilution conditions were established under which the magnitude of MLR proliferative response to Mls determinants was proportional to the number of responding T cells. Experiments carried out under such conditions demonstrated that T cells do not respond to Mls determinants alone, but rather that distinct T cell subpopulations exist which recognize Mls in the context of "self" H-2 determinants.

CTL were generated by the response of B6 ($H\text{-}2^b$) spleen cells against stimulating cells differeing from B6 only by point mutations in the K^b region. These responses were compared with the responses of $H\text{-}2^b \rightarrow H\text{-}2^d$ or $H\text{-}2^d \rightarrow H\text{-}2^b$ chimeras to K^b mutant cells or entirely allogeneic stimulators.

The responses to selected K^b mutants were strictly determined by the environment in which responding T cells had matured, so that normal $H\text{-}2^b$ or $H\text{-}2^d \rightarrow H\text{-}2^b$ chimeric cells generated strong CTL responses, while $H\text{-}2^b \rightarrow H\text{-}2^d$ responding cells were selectively unresponsive.

Significance to Biomedical Research and the Program of the Institute: The mixed lymphocyte response provides a useful model for T cell recognition as well as an in vitro correlate of allograft rejection. Further understanding of the recognition process as well as the primary stimulator cell should provide insight into controlling or preventing allograft and/or tumor challenges.

Proposed Course of Project: Specific anti-H-2 and anti-Ia reagents purified from hybridoma cell lines are being evaluated for their ability to block MLR responses to either H-2 or M1s differences. In addition, further work is in progress to study the mechanism by which host environment determines the alloreactive T cell repertoire.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05088-03 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effects of Graft Vs. Host Reactions on Cell-Mediated Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
Other:	U. Hurtenbach	Visiting Associate	I NCI
	J. Chalmer	Visiting Fellow	I NCI
	T. Tsuchida	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The intravenous injection of F_1 hybrid mice with parental spleen cells resulted in a loss in the ability of the F_1 mice to generate T-cell mediated cytotoxic responses in vitro to TNP-self and alloantigens. The loss of response potential depended on the H-2 type of the parental cells, since H-$2^{k,a}$ spleen cells induced unresponsiveness, whereas H-2^b spleen cells did not. The phenomenon is: (a) strain-specific (possibly depending on the C57Bl/10 genetic background), since strains of mice with other genetic backgrounds injected with parental cells do not exhibit the loss of immune potential; and (b) dependent on a reaction against F_1 alloantigens by grafted parental cells (GVH), since loss of immune activity was associated with enlarged F_1 host spleens. Suppressor cells were found to be responsible for loss of immune potential. The failure of lymphocytes from parental strains was shown to be due to F_1 resistance to parental T cells, which mapped to H-$2D^b$. Protection against GVH-associated suppression could be achieved using anti-H-2 sera directed against specificities of donor or host.

Project Description

Objectives: The purpose of this project is to investigate the phenomenon of immunosuppression induced by or associated with a graft vs. host reaction, the immunogenetics associated with the resistance in some strain combinations of the graft vs. host associated suppression, and to attempt to establish whether this immunosuppression is correlated with autoimmune or neoplastic states following a graft vs. host reaction.

Methods Employed: F_1 hybrid mice of various strain were injected intravenously with from 1 to $40x10^6$ F_1, parental, or allogeneic spleen cells. At various times after injection, the spleens of the injected F_1 mice were sensitized in vitro against: (a) parental or F_1 syngeneic cells modified with TNBS; or (b) allogeneic spleen cells. The effector cell actively generated 5 days later was tested on the appropriate ^{51}Cr-labelled target cells. Mapping studies were performed using inbred and recombinant mice on the C57BL/10 genetic background, as well as strains of other genetic backgrounds. Mice were injected with anti-sera or monoclonal reagents specific for H-2 region and subregion gene products. These reagents were supplied by the laboratory of Dr. D. H. Sachs.

Major Findings: F_1 hybrid mice on the C57BL/10 genetic background injected intravenously with viable parental spleen cells lost their ability to respond by in vitro generated cytotoxic reactions to TNP-self and alloantigens. The loss of cytotoxic potential was detected as early as four days after injection and persisted for at least 30 days after injection. Recovery from immunosuppression was detected 40-45 days after injection of parental cells. The abolition of cytotoxic potential: (a) appeared to depend on a graft vs. host reaction by parental lymphocytes against host alloantigens; and (b) was dependent on the H-2 haplotype of the parental cells used, since the injection of B10.A or B10.BR but not C57BL/10 (B10) parental cells resulted in loss of immune reactivity. The latter observation indicated that the B10 parental cells injected were rejected by the F_1 anti-parent reaction known as hybrid histoincompatibility (Hh). Genetic studies indicate that the $H\text{-}2^b$ homozygous determinant recognized by the F_1 maps to the H-2D region, which is compatible with an Hh-like phenomenon. The abolition of cytotoxic potential by the GVH reaction is the result of an active suppressive mechanism, since the addition of spleen cells from parental-injected F_1 mice to normal F_1 spleen cells led to the inactivation of the cytotoxic potential of the normal cells. The GVH associated immunosuppression may strain dependent, and may require non-H-2-linked genetic factors associated with the C57BL background, since mouse strains not on the C57Bl background injected with parental spleen cells did not result in suppressed immune potential. Protection against suppression was observed in F_1 mice injected with anti-H-2 antibodies specific for K, I, or D region gene products expressed either by the F_1 host or parental donor. Such protection was observed: (a) by using either anti-H-2 sera or monoclonal reagents, and (b) by injecting either F_1 host or parental donors. It was also found that the induction of suppression requires recognition of I region determinants expressed by the F_1 by the parental spleen cells.

Significance to Biomedical Research and the Program of the Institute: The graft vs. host (GVH) reaction and possibly Hh-type reactions are important complicating factors which affect the success of hemopoietic transplantation. Furthermore, persisting GVH reactions may be associated with autoimmune disease and the development of tumors. The observations: (a) that GVH reactions can be elicited with low numbers of lymphocytes in immunocompetent adult mice (previous reports have been limited to the demonstration of GVH in neonates or immunosuppressed animals); (b) that these GVH reactions lead to severely impaired T-cells immune functions; and (c) that such GVH reactions can be overcome by host resistance mechanisms are potentially of fundamental relevance in: (1) understanding the possible complications resulting from hemopoietic grafting; (2) investigating the significance of a GVH-induced suppressed immune system in the development of autoimmune and neoplastic disease; and (3) understanding natural resistance systems as they may be relevant in surveillance against disease and neoplasms.

Proposed Course of Project: We shall continue to investigate all aspects of the phenomenon including: (a) the genetics of the F_1 and parental cells involved; (b) the determinants recognized on the F_1 cells; (c) the mechanistic aspects of both the GVH and the suspected Hh component involved; (d) other immune functions which may be impaired including antibody production, delayed hypersensitivity, skin graft rejection, T-cell proliferative responses, and natural killer cell activity; (e) the long-term effects of the GVH including survival and the development of autoimmune disease and tumors; (f) whether certain combinations of partially allogeneic (instead of F_1 and parental) cells and hosts can lead to GVH-associated immunosuppression; (g) whether the GVH reaction is actually a component of the impaired immune state; (h) whether haplotype-specific anti-T-cell receptor suppression can be induced in the F_1 parent combination; and (i) analysis of different populations of cells involved in the induction of and protection by antibody against GVH-associated immunosuppression.

Publications

Shearer, G. M. and Polisson, R. P.: Mutual recognition of parent and F_1 lymphocytes: Selective abrogation of cytotoxic potential of F_1 lymphocytes by parental lymphocytes. J. Exp. Med. 151: 20-31, 1980.

Shearer, G. M., Polisson, R. P., Miller, M. W., and Cudkowicz, E.: Genetic control of natural resistance to graft versus host-associated suppression to T cell-mediated lympholysis. In Skamene, E., Kongshavn, P. and Landy, M. (Eds.): Genetic Control of Natural Resistance to Infections and Malignancy. New York, Academic Press. pp. 485-494, 1980.

Polisson, R. P. and Shearer, G. M.: Mutual recognition of parent and F_1 lymphocytes. II. Analysis of graft versus host-induced suppressor cell activity for T-cell mediated lympholysis to trinitrphenyl-self and alloantigens. J. Immunol. 125: 1855-1861, 1980.

Shearer, G. M. and Polisson, R. P.: Mutual recognition of parental and F_1 lymphocytes. III. Parental determinants recognized by F_1 host mice in resistance to graft-versus-host-associated immunosuppression map to H-$2D^b$. J. Immunol. 126: 545-547, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05090-03 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Role of Accessory Cells in B Cell Activation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Morrissey	IPA Investigator	I NCI
Other:	A. Singer	Senior Investigator	I NCI

COOPERATING UNITS (if any) I. Scher, Department of Medicine, Uniformed Services School of Medicine and Department of Experimental Pathology, Naval Institute of Medical Research, Bethesda, MD and A. Ahmed, Merck Institute for Therapeutic Research, Rahway, NJ

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A novel insight into the role of macrophage accessory cells in B cell activation was provided by the observation that the immune response to the thymic independent (T-I) antigen, TNP-Ficoll, was dependent upon the presence and function of splenic adherent accessory cells. The functional spleen adherent cell was identified as a phagocytic macrophage-like cell which bears the I-A gene product. The accessory cell for this T-I TNP-Ficoll response functioned to present antigen to B cells. This identifiable macrophage-B cell interaction was found to be obligately restricted to the Lyb 5+ B cell subset which appears late in normal B cell ontogenetic development and which is absent in hybrid mice which have received the X-linked immune deficiency gene of the CBA/N mouse. The existence of this important cell interaction required for activation of B cells in response to accessory cell dependent antigens such as TNP-Ficoll (T-I) and the thymic dependent (T-D) TNP-KLH provides explanation for the immune response deficit to the T-I and T-D antigens in mice which bear the X-linked CBA/N genetic defect.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The major objectives of this project are: 1) to identify the specific immune function and the cell surface phenotype of the macrophage-like accessory cell important for immune responses to thymic dependent and thymic independent antigens; 2) to investigate the mechanism of B cell activation mediated by direct interaction of B cells with antigen presenting accessory cells.

Methods Employed: Spleen cells from normal or immune defective mice are dispersed into single cell suspensions and placed into microculture with TNP-modified antigens (KLH, Brucella abortus, Ficoll) for 4 days. The cells are then harvested and assayed for IgM anti-TNP antibody forming cells by the slide modification of the Jerne plaque technique.

Splenic B cells are obtained by treating spleen cells with a T-cell specific rabbit anti-mouse brain and complement. Subpopulations of B cells are obtained based on their expression of a non H-2 encoded cell surface antigen, Lyb 5. CBA/N B cells constitute a pure population of immature B cells which lack the marker Lyb5. Treatment of normal B cells with an alloantiserum, anti-Lyb 5 and complement results in deletion of the mature Lyb 5 positive population, thus creating the CBA/N defect in normal spleen cells. Splenic T cells are obtained by passage over a nylon wool column. Splenic adherent cells are obtained by allowing spleen cells to adhere to glass petri dishes for 2 hours, discarding the nonadherent cells, and finally collecting the adherent cells by the addition of EDTA. Spleen cells are depleted of macrophages by passage over a G10 sephadex column. In some experiments the ability of splenic macrophages to phagocytose fluorescent latex beads was utilized to allow purification of phagocytic and non-phagocytic populations using the fluorescence activated cell sorter (FACS).

Major Findings: The in vitro response to TNP-Ficoll, a polysaccharide T-I antigen was dependent upon the presence of adherent accessory cells since passage of spleen cells over G-10 Sephadex columns prior to culture abolished the response. The required accessory cell was both adherent and phagocytic since responses depleted by either spleen cell passage over G-10 Sephadex or by removal of cells phagocytosing fluorescent latex on the FACS, could be fully reconstituted both by addition of splenic adherent cells or by the phagocytic fraction of adherent cells. In addition, SAC could be pulsed with TNP-Ficoll to present the antigen to activate B cells to form antibody. Treatment of normal spleen cell populations with anti Lyb5, like passage of cells over G10 Sephadex, abrogated TNP-Ficoll (T-I) and TNP-KLH (T-D) responses. This effect was not a result of the cytotoxic deletion of macrophages but, rather, of the deletion of B cells. Thus, the macrophage functioning in T-I, as well as T-D responses has the phenotype Ia^+, Lyb 5^-. The fact that responses requiring adherent accessory cells, such as the T-D TNP-KLH and the T-I TNP-Ficoll responses, emanate entirely from Lyb 5^+ B cells, suggested that only Lyb 5^+ B cells respond to macrophage activation signals. This possibility was validated by the demonstration that TNP-BA, a T-I antigen which activates both Lyb 5^- and Lyb 5^+ B cells when added directly to culture, activated

only Lyb 5^+ B cells when presented by pulsed macrophages. This concept of restricted activation of Lyb 5^+ B cells by macrophages provides important insight into the X-linked immune deficiency of the CBA/N mouse because this mouse lacks the Lyb 5^+ B cell subset and exhibits immune deficiency to macrophage dependent antigens.

Significance to Biomedical Research and the Program of the Institute: Utilization of techniques for separation of splenic lymphocyte and macrophage populations has facilitated precise understanding of the cellular interactions involved in the normal immune response, and the abnormalities of these interactions in a genetic immune deficiency. Such understanding is the primary step to allow for the possibility of alteration of imbalanced immunity as it appears in clinical medicine.

Proposed Course of Project: Further work will be directed to the delineation of of the nature of the signals transmitted from macrophages to T and B lymphocytes populations to effect an immune response.

Publications

Boswell, H. S., Ahmed, A., Scher, I., and Singer, A.: Role of accessory cells in B cell activation. II. The interaction of B cells with accessory cells results in the exclusive activation of an Lyb5+ B cell subpopulation. J. Immunol. 128: 1340-1348, 1980.

Boswell, H. S., Nerenberg, M. I., Scher, I., and Singer, A.: Role of accessory cells in B cell activation. III. Cellular analysis of primary immune response deficits in CBA/N mice: Presence of an accessory cell-B cell interaction defect. J. Exp. Med. 152: 1194-1209, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05091-03 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Target Antigen Recognition by Cytotoxic T Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.A. Henkart	Senior Investigator	I NCI
Other:	H. Pehemberger	Fogarty Fellow	I NCI
	G. M. Shearer	Senior Investigator	I NCI
	R. B. Levy	Senior Staff Fellow	I NCI
	K. Ozato	Visiting Associate	I NCI
	D. H. Sachs	Acting Chief	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.8	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1)Plasma membrane vesicles were prepared from tumor cells which are good target cells for cytotoxic T lymphocytes (CTL); by comparing their interaction with CTL before and after fusion of the vesicle membrane with the membrane of a tumor cell not recognized by the CTL, it was concluded that insertion of the H-2 antigen recognized into a living cell membrane greatly enhances recognition by CTL. (2)H-2 antigens on lymphoid cells were studied in binding experiments with labeled monoclonal anti-H-2. One example of a single H-2 antigen having two distinct determinants was found. (3) Spleen cells modified by several haptenic -SH reagents were found to stimulate the production of H-2 restricted hapten-specific CTL after in vitro culture with syngeneic spleen cells. Comparison with previously developed hapten modified CTL recognition systems shows the new system to be more H-2 restricted; the recognized hapten is on an -SH group not part of H-2. Epidermal cells modified with these reagents could also stimulate such CTL.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To understand the molecular mechanisms involved in the binding of cytotoxic T lymphocytes to the target cells which they specifically recognize. This is being attempted by several approaches: (1) To develop a means of quantitatively studying the binding reaction without looking at cytotoxicity; (2) To better understand the qualitiative and quantitative display of transplantation antigens on the membrane of a variety of cells, since these antigens apear intimately involved in the recognition of all types of target cells by CTL; (3) To develop new and better defined systems for H-2 restricted killing of hapten-modified target cells.

Methods Employed: (1) Plasma membrane vesicles are prepared from RDM-4 tumor cells ($H\text{-}2^k$) by the nitrogen decompression method. Such vesicles were adhered to P388D1 ($H\text{-}2^d$) tumor cells by wheat germ agglutinin, and in some cases fused with 40% polyethylene glycol. Alloreactive cytotoxic T lymphocytes are obtained from secondary in vitro cultures of $H\text{-}2^b \times H\text{-}2^d$ spleen cells with irradiated $H\text{-}2^k$ spleen cells. (2) Purified monoclonal anti-H-2 antibodies are labeled with ^{125}I and allowed to bind to several million spleen cells. Bound antibody is detected by spinning the cells through oil and counting them in a gamma counter. (3) The most utilized reagent for modifying cell surface SH groups is 1,5-iodoacetylethylenediaminenapthylene sulfonic acid (I-AEDANS). The cells are modified by treatment with 1mM reagent; pH 8, 37° for 30 minutes. Standard CML culture conditions are used to generate CTL, except that cultures are carried out for 7 days, and $H\text{-}2^b$ is the high responder. ^{51}Cr labeled target cells are modified by the above procedure. Anti-AEDANS was made in rabbits by injection of a KLH conjugate. Affinity-purified anti-AEDANS was FITC labelled and used with the FACS to quantitate cell-surface hapten groups. That the CTL recognize AEDANS on -SH groups was shown by pre-treatment of the target cells with other SH reagents prior to the AEDANS reaction. That H-2 is not the recognized AEDANS modification was shown by co-capping and the use of H-2 mutants. Epidermal cells are prepared by dissecting off the body skin of appropriate mice and dissociating the cells using trypsin.

Major Findings: (1) Our studies have established that recognition of membrane vesicles as such by CTL is inefficient. If vesicles are bound to the surface of ^{51}Cr labelled target cells not bearing antigens recognized by the CTL, no target cell lysis occurs. However, after fusion of the vesicle membranes into the target cell membrane with PEG, these cells are lysed by the CTL. Thus some aspect of the living cell membrane confers "recognizability" to the H-2 antigens. (2) Labelled monoclonal anti-$H\text{-}2K^k$ antibodies were shown to bind specifically to living cell membranes. Such binding was shown to be inhibitable with cold anti-$H\text{-}2K^k$ antibodies, and one combination of two monoclonal antibodies were found to bind non-competitively. This was interpreted as showing two spatially separate sites on the antigen recognized by specific antibodies. (3) Cell surface -SH groups were modified with haptenic -SH reagents; such cells stimulated the production of H-2 restricted, hapten specific CTL when cultured in vitro with syngeneic spleen cells. It was shown that CTL recognize modified -SH groups on the membrane which are not on H-2, there are about 10^4 haptens/cell, and that recognition can be

specifically blocked by anti-hapten antibody. This "modified-self" system is significantly more H-2 restricted than lysine-reacting haptens like TNP. In an effort to detect CTL restricted by hapten-modified differentiation antigens, epidermal cells modified with such haptens were found to induce CTL when used as stimulator cells in the in vitro culture; in vivo priming of the responder cells was required. Thus far, no epidermal-specific component of the recognition has been detected, since both lymphoid and epidermal cells are lysed by these CTL.

Significance to Biomedical Research and the Program of the Institute: T lymphocytes play a central role in most immune responses and much recent speculation about their antigen receptors has resulted from inferences from complex functional experiments. We hope to devise ways of studying this recognition process by carrying out more chemically defined studies of the binding process. An understanding of this receptor's interactions will not only aid in a basic understanding of the process of CTL killing, but also serve as a model for all T-cell-cell interactions in the immune system.

Proposed Course of the Project: Methods are being tried out to improve the efficiency of membrane fusion. We are currently carrying out quantitative analyses of H-2 antigens on cell membranes. Methods are being devised to short-circuit the normal recognition receptors using chemically defined cross-linking between CTL and target cell. Cloned CTL will be used to eliminate the heterogeneity in the currently used populations.

Publications

Levy, R.B., Shearer, G.M., Richardson, J.C., and Henkart, P.A.: Cell mediated lympholytic responses against autologous cells modified with haptenic sulfhydryl reagents. I. Effector cells can recognize two distinct classes of hapten-reactive self sites on cell surface proteins. J. Immunol., in press.

Levy, R.B., Henkart, P.A., and Shearer, G.M.: Cell mediated lympholytic responses against autologous cells modified with haptenic sulfhydryl reagents. II. Analysis of the genetic control of cytotoxic responses to sulfhydryl and amino reactive reagents. J. Immunol., in press.

Ozato, K., Henkart, P.A., Jansen, C., and Sachs, D.H.: Spatially distinct allodeterminants of the H-2K^k molecule as detected by monoclonal anti-H-2 antibodies. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05092-02 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Cellular Expression of Idiotypic Markers and Role in Immune Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.I. Nadler	Investigator	I NCI
Others:	G. Miller	Investigator	I NCI
	D.H. Sachs	Chief, Transplantation Biology Section	I NCI
	R. J. Hodes	Chief, Immunotherapy Section	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION Immunotherapy and Transplantation Biology Sections

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The antibody and T cell proliferative responses to Staphylococcal nuclease, a small peptide antigen, have been shown to be under Ir gene control. In addition anti-idiotypic antisera have been produced with specificity for anti-nuclease antibodies of a number of mouse strains. It has been demonstrated that these idiotypic markers are linked to allotype markers but not to MHC encoded (H-2) determinants. The present study has investigated the cellular expression of idiotypic markers in this Ir gene controlled response. In vitro augmented primary responses to TNP-nuclease could be generated after priming with nuclease (antigen), anti-idiotypic antisera (Ab2), or anti-[anti-(anti-idiotype)] (Ab4). These responses to TNP-Nase were shown to be inhibited by anti-idiotype present in culture. It was demonstrated that antigen, anti-idiotype or Ab4 primed T cells were required and that treatment of the primed T cells with anti-idiotype and complement abrogated their helper cell activity. Thus, direct evidence for helper T cell expression of idiotypic markers has been obtained although it is as yet unclear whether the T cell synthesizes or acquires these cell surface determinants. Studies with nuclease specific T cell clones are under way to further elucidate the nature of helper T cells and their antigen-specific receptors.

Project Description

Objectives: The expression of V_H region markers (idiotypes) on sIg molecules of B cells is well established. There has been considerable controversy regarding the existence of similar V_H encoded idiotypic determinants on T cell subpopulations. The present studies were undertaken to examine the expression of idiotypic markers on T cells participating in vitro antibody responses to TNP-Nuclease.

Methods Employed: An in vitro augmented primary response to TNP-Nuclease was developed in which accessory cells, unprimed B cells, and T cells primed with Nuclease (Ag), anti-nuclease anti-idiotype (Ab2) or anti-[anti-(anti-idiotype)] (Ab4) were required. IgM plaque forming cell responses to TNP-Nuclease are antigen-dependent and TNP-specific. Standard procedures for preparation of B cells (G-10 Sephadex passage and RAMB+C treatment), T cells (nylon column passage), accessory cells (glass adherence, irradiation, and RAMB+C treatment), and accessory cell depleted spleen cells (G-10 Sephadex passage) were employed. Anti-idiotypic antisera were prepared and purified as described in Project No. Z01-CB-05036-08 I. Functional competence of spleen cell subpopulations after treatment with anti-idiotypic antisera + C was assessed. An ELISA (enzyme linked immunoadsorbent assay) was developed to measure anti-nuclease antibodies in serum and/or culture supernatants. This assay was also adapted for detection of anti-idiotypic antibodies which inhibit anti-Nase antibody binding to antigen (Nase) coated plates, and for detection of Nase idiotype in serum by inhibition of anti-idiotype binding to idiotype coated plates.

Major Findings: (1) In vitro TNP-specific IgM augmented primary responses to TNP-nuclease could be generated after priming spleen cells with either nuclease (Ag), anti-nuclease anti-idiotype (Ab2) or anti-[anti-(anti-idiotype)] (Ab4). These responses required primed T cells, B cells, and accessory cells.

(2) This in vitro augmented response to TNP-nuclease was shown to be under Ir gene control paralleing the control of the in vitro T cell proliferative response to nuclease and the in vivo antibody response to nuclease (see Project No. Z01-CB-05036-08 I and Z01-CB-05064-04 I). Accessory cells for these in vitro responses to TNP-Nase were shown to express the Ir gene defect.

(3) In vitro PFC responses to TNP-nuclease of nuclease, anti-idiotype (Ab2) or Ab4 primed BALB/c spleen cells were inhibitable by the presence in culture of anti-idiotypic antisera specific for a pool of BALB/c anti-Nase antibodies but not by anti-idiotypic antisera to SJL anti-Nase or normal pig immunoglobulin. Cell fractionation and mixing experiments have demonstrated that this inhibition occurs at the level of helper T cells.

(4) Treatment of nuclease, anti-idiotype, or Ab4 primed splenic T cells with specific anti-idiotype + C markedly diminished or abrogated the ability of these cells to provide helper cell function for in vitro responses to TNP-nuclease. Treatment of Nuclease primed B10.D2 spleen cells with anti-BALB/c anti-idiotype + C had little or no effect on their helper cell function. In addition, treatment of nuclease primed BALB/c T cells with either specific anti-SJL anti-idiotype or pig anti-BALB/c Ig+C did not affect T_H function for anti-TNP-nuclease responses.

(5) Priming of congenic high and low responder A/J and A.BY with anti-idiotype resulted in the development of idiotype-bearing, non-antigen binding molecules (ID') in both but did not circumvent the Ir gene defect of the $H\text{-}2^b$ mice for in vitro response to TNP-Nase.

(6) Treatment of B10.D2 ($H\text{-}2^d$, $Igh\text{-}C^b$) mice with either Nase or anti-BALB/c ID resulted in priming for in vitro responses to TNP-Nase. These B10.D2 mice primed with Nase expressed idiotypes normally expressed on B10.D2 antibodies in response to Nase on their helper T cells. Anti-BALB/c ID primed B10.D2 helper T cells expressed BALB/c ID determinants on their surface.

Significance to Biomedical Research and the Program of the Institute: The development of a new system in which the cellular expression of idiotypic determinants may be investigated for a response which is also under Ir gene control may be useful in investigating both the mechanism of Ir gene function and the regulation of immune responses by the proposed idiotype- anti-idiotype network. The major thrust of this endeavor has been aimed at delination of the T cell antigen receptor and its relation to the more well-characterized B cell receptor, surface immunoglobulin. Anti-idiotypic antisera recognizing determinants (V_H region encoded) linked to allotype have been used both to activate T cells in vivo for in vitro TNP-Nuclease responses and to determine whether the T helper cells required for in vitro TNP-nuclease responses bear idiotype. These demonstrations of idiotypic determinants on helper T cells similar or identical to B cell determinants should permit further characterization of the antigen receptor of T cells both functionally and chemically.

Proposed Course of Project: Studies are underway utilizing clones of nuclease primed T cells to asssess these cells for T helper cell function and expression of idiotype. In addition, attempts will be made to assess whether these idiotype-bearing T_H cells are MHC restricted or whether idiotypic restrictions exist within this system.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05093-02 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Environmental Influences on Self-Tolerance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.J. Morrissey	IPA Investigator	I NCI
Other:	A Singer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The influence of the prethymic compartment on the acquisition of tolerance to major histocompatibility antigens in the mouse was evaluated by altering this prethymic environment. Thus, strain A bone marrow cells were injected into a lethally irradiated $(AxB)F_1$ mouse. At a later time, the bone marrow from this $(AxB)F_1$ mouse was isolated and re-introduced into a lethally irradiated strain A host. Under conditions which are not now completely understood temporary tolerance to the B haplotype was achieved. To further investigate, the influence of the prethymic environment on the acquisition of tolerance to MHC antigens, $(AxB)F_1$ mice were thymectomized then grafted with a strain A thymus and later irradiated and reconstituted with strain A bone marrow. The engrafted thymus was later tested for the presence of cells bearing the B-haplotype and for reactivity toward this determinant. Preliminary results indicate that although the B haplotype is not present in the thymus, tolerance exists. Therefore, alloreactive patterns in the thymus may be affected by environmental manipulation of the pre-thymic compartment.

Project Description

Objective: These experiments will study the role of different cellular compartments on the development of tolerance and self recognition in chimeric mice.

Methods Employed: Young adult mice, strain (AxB)F_1 were thymectomized and later grafted subcutaneously with thymus lobes obtained from a newborn strain A mouse. Three to five days later, these mice were lethally irradiated and reconstituted with T-cell depleted bone marrow from strain A mice. Starting four weeks later these mice were sequentially studied at weekly intervals. The grafted thymus was isolated and the cells analyzed on the FACS II for the presence of the B haplotype. These cells were also tested for their alloreactivity and self-recognition patterns by the in vitro generation of cytotoxic lymphocytes and proliferation in a mixed lymphocyte reaction.

Major Findings: In thymectomized mice of strain (AxB)F_1 which have been grafted with a strain A thymus, irradiated, and given strain strain A bone marrow, tolerance was found to the B haplotype in the thymus in the absence of any cells bearing that haplotype. Thus, in a situation in which alloreactivity to B would be expected to arise (strain A thymocytes in a strain A thymus) it does not. Therefore the tolerizing influence must occur earlier than the thymus and also prethymocytes must express receptors for antigens prior to entry in the thymus.

The patterns of self-recognition in these mice are also being evaluated using TNP modified self as a stimulator for the generation of cytotoxic lymphocytes. Preliminary results indicate that recognition of TNP modified cells of the B-haplotype exists, but it is not yet known if this observation is a result of the degeneracy of the anti-A TNP response or actually represents restricted self recognition.

Significance to Biomedical Research and the Program of the Institute: Insights into the mechanism of self tolerance are important for our understanding of the function immune system and the generaton of the T-cell repertoire. As the underlying principles become unraveled, it is hoped that they will have a significant impact on human transplantation medicine and the immunological approach to treating cancer.

Proposed Course of Project: The project will continue to investigate the role of the bone marrow environment in self tolerance in depth. Also, the relationship between tolerance and self recognition in these chimeras will also be investigated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05094-02 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Role of the Thymus in Generation of the Self-MHC Specific T Cell Repertoire

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Kruisbeek	Guest Worker	I NCI
Other:	A. Singer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to assess the role of the thymus in the acquisition of the T cell self-MHC specific repertoire. The MHC specificity of cytotoxic precusor T cells differentiating within thymuses engrafted into athymic nude mice was determined. The results demonstrated that cytotoxic T lymphocytes from such engrafted thymuses recognized TNP in association with thymic MHC determinants in various haplotype combinations of thymus graft and nude host. In contrast, cytotoxic T lymphocytes from the spleens of these same thymus grafted nude mice recognized TNP on both nude host and thymic MHC determinants. These results are consistent with the thymus being one site which determines MHC restrictions of T cell self recognition, while there exist other factors in this experimental model which have determined the host-restricted MHC specific repertoire observed in the spleens of thymus engrafted nude mice.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The objective of this project is to assess the presumed role of the thymus in determining the recognition specificities of killer T cells directly by measuring the killer T cell responses to foreign antigens which are recognized in the context of H-2 within the thymus, rather than in the periphery.

Methods Employed: Thymocytes generally give low killer T cell responses to alloantigens and TNP-modified self H-2 antigens. Application of T cell growth factor within the cultures however allows thymocytes to express strong killer T cell responses with maintenance of specificity for the original stimulating signal. Thus, a method is available to study the specificity repertoire of thymocyte T killer cells. Complicating allogeneic effects are avoided by using radiation bone marrow chimeras and thymus-engrafted nude mice.

Major Findings: In both radiation bone marrow chimeras and thymus-engrafted nude mice, thymocyte T killer cells display recognition of TNP in association with thymic MHC determinants only. These results indicate that the recognition pattern observed in the thymus is determined by the MHC phenotype of the thymus. In fully allogeneic nude host-thymus graft haplotype combinations, such a thymus-determined recognition pattern is also observed in the spleen; however, in addition to self-recognition of thymic MHC determinants, host MHC determinants are also used as self-recognition elements. This suggests that in this model, 2 different sites, i.e., the engrafted thymus and an unknown host element, have been operational in determining the self-MHC specific repertoire of splenic cytotoxic T lymphocytes, while the thymic repertoire is determined solely be the thymic MHC determinants.

Significance to Biomedical Research and the Program of the Institute: The above project will allow a better insight into the mechanisms responsible for the regulation of T lymphocyte responses to foreign antigens and thereby yield ways to manipulate immune disorders in humans which are a consequence of regulatory failure.

Proposed Course of Project: The project will next investigate whether also in other experimental modesl, besides a thymus-determined repertoire, an extra-thymically determined T cell self-MHC specific repertoire can be detected.

Publications:

Kruisbeek, A. M., Hodes, R. J., and Singer, A.: Cytotoxic T lymphocyte responses by chimeric thymocytes: Self-recognition is determined early in T cell development. J. Exp. Med. 183: 13-29, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05095-02 I

PERIOD COVERED

October 1, 1980 to September 30, 198

TITLE OF PROJECT (80 characters or less)

Regulation of Cell-Mediated Immunity by Germ Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	U. Hurtenbach	Visiting Associate	I NCI
Other:	G. M. Shearer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER x

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Autologous mouse testicular cells derived from the seminiferous tubules activate suppressor T cells which inhibit a syngeneic or allogeneic mixed cell reaction in vitro. Generation of cytotoxic T cells in vitro is reduced in the presence of syngeneic germ cells (spermatozoa from the siminiferous tubules or epididymal sperm). Spleen cells from mice injected with syngeneic sperm show a nonspecifically suppressed potential to generate cytotoxic T cells in vitro. In contrast, spleen cells from mice injected with syngeneic testicular cells derived from the seminiferous tubules exhibit a reduced response against TNP-modified self antigen; spleen cells from mice inoculated with allogeneic testicular cells from the seminiferous tubules suppress the cytotoxic response against splenic stimulators of the same haplotype.

Project Description

Objectives: The objective of this study is to investigate the antigenic, genetic and cellular requirements leading to an autoimmune reaction against male germ cells. For this purpose, murine male germ cells have been chosen as antigen. Due to their morphological isolation, immunological tolerance is not acquired, and thus their cells remain autoantigenic.

Methods Employed: Sequential protease treatment of murine testes released two fractions of cells: a population consisting of interstitial cells; and a second population which is derived from the seminiferous tubules consisting of spermatozoa. Sperm cells were obtained from the epididymis. Spleen cells from normal mice of various strains were sensitized in vitro against autologous spleen cells modified with a trinitrophenyl group or against allogeneic stimulators in presence of autologous germ cells. In another protocol spleen cells from mice previously injected with germ cells were stimulated in vitro against modified syngeneic or allogeneic spleen cells. Lymphocyte reactivity was assayed by measuring the proliferative response by ^{3}H thymidine incorporation and by determining cytotoxic activity on ^{51}Cr-labelled PHA blast cells.

Major Findings: The two testicular cell populations showed different effects by lymphocyte proliferation in vitro: lymphocyte reactivity was suppressed against the autologous germ cell population itself as well as against autologous or allogeneic interstitial cells or against allogeneic spleen cells when germ cells were present during the sensitization phase. In contrast, the testicular cells consisting of an enriched population of interstitial cells stimulated lymphocyte proliferation. The reactive lymphocytes were T cells; suppression could be abrogated by treatment of the responder cells with anti-Ly 2.2 sera, plus complement. Lymphocyte proliferation was significantly reduced by anti Thy 1.2 plus complement treatment. Similar suppressive effects of autologous germ cells have been observed on the generation of cytotoxic T lymphocytes in vitro. In the presence of spermatozoa from the seminiferous tubules the reactivity against modified self or alloantigen was reduced, whereas interstitial testicular cells had no significant effect. No difference has been found in responder spleen cells between male or female mice.

Spleen cells from young adult male mice injected i.v. with syngeneic sperm and then followed by sensitization in vitro against splenic stimulators exhibited strongly reduced potential to generate cytotoxic T lymphocytes against modified self and to a lesser extent, against alloantigens. Co-cultivation of these spleen cells with normal responder cells showed that the lack of cytotoxic reactivity was due to a suppressor mechanism. Suppression was more pronounced with increasing age of the sperm donor mice. The affected recipients exhibited symptoms of graft vs. host disease of various degrees. So far, haplotype restriction has not been found. Injection of germ cell from the seminiferous tubules (which contain immature spermatozoa of various differentiation stages) followed by in vitro sensitization against splenic stimulation also showed

reduced cytotoxic potential. However, in contrast to the unspecific suppression induced by syngeneic sperm, the testicular cell induced suppression was specific. Thus, injection of syngeneic testicular cells inhibited the generation of a CTL response against modified-self only, whereas injection of allogeneic testicular cells exclusively inhibited the response against the stimulators of the inoculated haplotype, but not against a third-party stimulator.

Significance to Biomedical Research and the Program of the Institute: Antigens have been shown to be expressed on cells of the male germ line as well as on tumors which derive from embryonic cells. Both cell types induced immunosuppression. Therefore, this project may be of medical relevance, since it may help to understand the immune status of individuals if such antigens come into contact with the immune system; e.g. after vasectomy or during development of neoplastic cells.

Proposed Course of Project: Experiments will be performed to investigate the mechanism leading to the germ cell-induced suppression of the CTL response. The antigenic determinants on the germ cells responsible for the induction will be investigated using monoclonal reagents. It will be tested (a) whether antibodies directed against specific surface structures bind to germ cells and (b) whether induction of suppression can be prevented by preinjection of the specifc monoclonal reagents. In addition, variation of antigen expression will be studied, since induction of suppression seemed to be related to the age of the germ cell donors. The target cell of the germ cell within the lymphoid cell population will be determined using physical or serological separation methods and consecutively their suppressive ability on normal spleen cells in co-cultivation experiments will be tested. Recombinant mouse strains will be used to determine whether there is genetic restriction at the level of the germ cells for induction of suppression and/or at the level of the interaction of the suppressor cells with the target lymphoid cell.

Due to the fact that another more interesting project is currently being pursued by the P. I. (See Z01 CB 05088) considerable efforts on this project have not been made during the past year. However, a manuscript is in preparation and it is anticipated that this project shall be completed in 1981-82.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05096-02 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Identification and Function of Intracellular Calcium-Containing Organelles

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. P. Henkart	Expert	I NCI
Others:	L. E. Waters	Biol. Lab. Tech.	I NCI
	C. E. Fiore	Physical Scientist	BEIB, DRS

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 21205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to determine the distribution of calcium in cellular organelles and the effects of physiologic stimuli on the distribution of calcium and the morphology of calcium-containing organelles. It is particularly concerned with the question whether the endoplasmic reticulum (ER) is a calcium uptake system and whether calcium can be released form the ER in response to surface membrane stimuli such as specific ligand-receptor interactions. Tissue is prepared by rapid freezing or by modified fixation procedures designed to retain Ca in situ. Ca is identified in sections by electron-probe x-ray microanalysis. Early stages of this study have been devoted to refinement and testing of the methods.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The general objective of this project is to study the distribution of calcium within cells and to determine how its distribution is affected by physiologic stimuli. A working hypothesis underlying a part of this study is that the endoplasmic reticulum (ER) of all cells can function as a calcium sequestering system similar to the sarcoplasmic reticulum of muscle. An implication of this hypothesis is that there may also be a mechanism by which calcium can be released from the ER in response to surface membrane stimuli, providing for a trans-membrane signal in many cell types analogous to excitation-contraction coupling in muscle.

Methods employed: Basic morphologic studies are done using standard techniques of electron microscopy. Identification of intracellular calcium-containing organelles require in addition: 1) Methods of tissue preparation that retain calcium in situ. Two approaches to this have been used: (a) In previous studies (in collaboration with Dr. T. S. Reese of NINCDS) cells were rapidly frozen and substituted with acetone in the presence of osmium. (b) Modified fixation procedures have also been used. These include fixation in aldehyde in the presence of oxalate followed by post-fixation in osmium in acetone. The results obtained by method (b) were compared with results obtained by method (a) using the squid giant axon and mouse skeletal muscle as test tissues. (2) Identification of clacium in the organelles of cells [prepared as in (1)]. This is done by electron-probe x-ray microanalysis using the analytic electron microscope facility being developed by BEIB in DRS.

Major Findings: In previous studies related to this problem I have shown that the endoplasmic reticulum of neurons is a calcium-sequestering compartment. In macrophages areas of both smooth and rough endoplasmic reticulum also contain calcium. The ER of macrophages forms morphologically specialized appositions (subsurface cisterns) with the surface membrane (or the membrane of newly internalized pinosomes) similar to the junctions between the surface membrane and sarcoplasmic reticulum of muscle at "triads". Other organelles also contain calcium. One general class includes organelles whose membranes circulate through the surface membrane via endocytosis and exocytosis. This class includes pinosomes and phagosomes, lysosomal structures and secretory granules. Some cisterns and vesicles in the vicinity of the Golgi apparatus also contain dense deposits, but these are vey small structures and it has not yet been possible to identify them in the images thus far obtained under conditions for x-ray analysis. Calcium has also been identified in the periphery of lipid droplets and in the space between the inner and outer mitochondrial membranes, but rarely is found in mitochondrial matrices.

Significance to Biomedical Research and the Program of the Institute: The role of calcium as a transmembrane signal or second messenger and its importance as a regulator of many intracellular functions is becoming increasingly apparent. Examples of calcium-regulated functions include: motility based on actin-myosin systems, secretion by exocytosis, control of membrane permeability to other ions, processes dependent upon polymerization of microtubules, the activity of many enzymes, and probably control of cell proliferation and differentiation. How calcium is distributed within cells and how its

distribution is affected by physiologic stimuli are, thus, questions of fundamental importance for the understanding of normal cell function. Although many studies have suggested that calcium may be important in control of cell growth, no unified hypotheses have emerged about mechanisms. Identification of calcium in its morphologic context may help to clarify some of the intricacies of cellular control of calcium and, thus, lay the foundation for future studies directed at the role of calcium in the cell biology of cancer.

Proposed Course of Project: Plans for this project still involve refinements of the techniques and determination of their limitations. Because of the failure of the supplier to deliver on schedule the apparatus required for rapid freezing, the development of this technique has been delayed. The analytic EM facility is also still under development. Until now the imaging capabilities of the BEIB microscope under conditions for x-ray analysis have been inadequate for identification of many of the organelles of interest. Further improvement of computer image processing is expected to provide a substantial improvement in imaging unstained material. When these techniques and facilities are available we expect to be able to continue with plans outlined in the annual report for 1979-80.

Publications

Henkart, M.: Identification and function of intracellular calcium stores in neurons. Introduction to Symposium. Fed. Proc. 39:2776-2777, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05098-01 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Non-H-2-linked genetic control of cell-mediated cytotoxic responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
	P. K. Arora	Visiting Fellow	I NCI
	H. Fujiwara	Visiting Associate	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Spleen cells from mice of different inbred strains sharing the same H-2 haplotype but differing in their non-H-2 genetic background were compared for their ability to generate cytotoxic T lymphocyte (CTL) responses to syngeneic cells modified with the trinitrophenyl hapten (TNP-self). In both primary and secondary responses, high and low CTL strains were observed (i.e. non-H-2-linked Ir gene control). Among H-2^d strains the BALB/c was a high responder strain, whereas DBA/2 and B10.D2 were low responder strains. Among H-2^k mice, C3H, AKR/J and B10.BR were the respective high , intermediate, and low responders. Of the H-2^b strains studied C57BL/6 were high, whereas C3H.SW and C57BL/10 were low responder strains to TNP-self. By using different combinations of responding, stimulating and target cells, it was found that these non-H-2-linked differences were not attributable to stimulating or target cells. These studies raise some interesting issues concerning the role of non-major histiocompatibility complex (MHC) genes in regulating CTL responses to foreign antigens recognized in association with self MHC gene products.

Project Description

Objectives: It has been previously demonstrated that H-2 linked genes which map to the K and D regions regulate the CTL responses to TNP-self. The objective of this project was to determine whether non-H-2 linked genes also have an effect on regulating the CTL response to TNP-self. If so, it is also the objective of this project to determine whether the thymus (which is known to influence the expression of H-2 linked genetic control of H-2 restricted CTL response) will affect non-H-2 linked Ir gene control of CTL.

Methods Employed: For in vitro generation of CTL, mouse spleen cells were sensitized in vitro to syngeneic cells conjugated with trinitrobenzene sulfonic acid (TNP-self), and the effector cells generated were assayed on the appropriate ^{51}Cr-labelled target cells. Mice whose spleen cells were to be used for in vitro sensitization to TNP-self were primed in vivo by skin painting with trinitrochlorobenzene.

H-2 matched allogeneic chimeras were prepared by irradiating recipient mice with 850R and by transferring bone marrow cells from donors, involving high responder recipients grafted with low responder stem cells and vise versa. Two-to-four months after cell transfer, the spleens of the chimeras will be tested in vitro for high and low response patterns to TNP-self.

Major Findings: Both in primary in vitro and in secondary in vitro (following in vivo priming) CTL studies the following non-H-2 linked high and low genetic control patterns were observed: among $H\text{-}2^d$ strains--Balb/C, high responder; DBA/2 and B10.D2, low responders; among $H\text{-}2^K$ strains--C3H, high responder; AKR/J, intermediate responder; B10.BR, low responder; among $H\text{-}2^b$ strains--C57BL/6, high responder; C34H.SW and C57BL/10, low responders. These differences were observed to be more pronounced in the secondary than in the primary response, and radioresistant helper T cells were demonstrated to be involved in at least part of the differences among high and low responder strains. By varying the strains used for providing responding, stimulating and target cells, it was found that the low responder patterns could not be accounted for by stimulating or target cell defects. Therefore, these difference in high and low responder strains are likely to reside among the helper, CTL precursor, and/or accessory cells provided by the responding cell pool.

Significance to Biomedical Research and the Program of the Institute: Over the last 15 years considerable emphasis has been placed on the importance of MHC linked Ir genes in the control immune responsiveness, and such regulation has been demonstrated both in experimental animal models and man. Based on the dramatic effects that the thymus has on both MHC restriction and on phenotypic expression of Ir genes, it has been postulated that Ir gene patterns of responsiveness are reflections of MHC restriction. The potential significance of the present project is that it demonstrates that Ir genes which are not linked to the murine MHC also have a dramatic effect on T cell

immune responses--even those which are MHC restricted. Such studies underscore the fact that in considering the genetic regulation of immune potential, heredity effects other than just those linked to the MHC must also be considered.

Proposed Course of Project: Among the strains thus far investigated, F_1 hybrids and backcross mice will be tested to establish whether high or low responsiveness is dominant and to obtain an estimate of the number of genes involved. Since these high and low responders are H-2 compatible, allogeneic irradiation chimeras are being prepared to determine whether high responsiveness is a characteristic of the host environment or of the donor stem cells. It may also be important to do thymic grafts in athymic nude mice to determine the role of the thymus in such Ir gene control. Congeneic mice differing at other known non-MHC markers will be compared to determine if there is linkage to other loci (e.g., allotype). CTL responses to other haptens plus self as well as to alloantigens will be investigated to determine how broad non-H-2-linked regulation of CTL responses are. Responding cell populations will be fractionated to attempt to define a particular cell population(s) which may express the genetic defect in low responder strains.

Publications

Fujiwara, H., and Shearer, G. M.: Non-H-2-associated genetic regulation of cytotoxic responses to hapten-modified syngeneic cells: Effect on the magnitude of secondary response and helper T cell generation after in vivo priming. Eur. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-05099-01 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunogenetic effects of murine cytomegalovirus on induced and natural immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	G. M. Shearer	Senior Investigator	I NCI
OTHERS:	J. Chalmer	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mice injected with sublethal doses murine cytomegalovirus (MCMV) exhibit rapid and dramatic changes in their ability to generate in vitro cytotoxic T lymphocyte responses to hapten-self and to alloantigens. Within three days after intraperitoneal injection of (MCMV), the CTL responses to hapten-self and alloantigens are abrogated or severely reduced. This is followed by rapid recovery to a normal level of CTL potential, and then to a hightened state of immune potential detected by the hapten-self CTL only. The injection of F_1 hybrid mice with either MCMV or parental spleen cells resulted in rapid and severe immunosuppression. Inoculation of either the virus or parental cells were selected so that they would be below the threshold for severe immunosuppression. However, when these two inocula were combined, severe immunosuppression was observed. These studies permit the investigation of the immunosuppression of MCMV infection and the possibility consequences of CMV infection coupled with a graft-versus-host reaction (GVHR).

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The purpose of this project is to investigate the immunological and genetic effects of MCMV infection and of MCMV infection plus a GVHR on (a) acquired T cell immunity to hapten-self antigens and alloantigens; and (b) on natural resistance to MCMV infection and the GVHR. Since certain mouse strains are relatively resistant to MCMV and to parental T cell-induced GVHR, it will also be the purpose of this study to investigate the role of H-2-linked and non-H-2-linked genetic effects of resistance and susceptibility to MCMV, to GVHR and to a combination of MCMV and GVHR.

Methods Employed: Sublethal doses of MCMV (prepared from salivary glands of infected mice) were injected intraperitoneally into various inbred and F_1 hybrid mouse strains. Also F_1 mice were injected intraveneously with known concentrations of parental spleen cells, and F_1 mice were also injected with MCMV plus parental cells. The T cell immune potentials of injected and control mice were tested by in vitro sensitization to hapten-self and allo-antigens, and the CTL activity was determined 5 days later using the ^{51}Cr-release assay.

Major Findings: The injection of sublethal doses of MCMV resulted in rapid suppression of CTL potential to both hapten-self and allogeneic antigens (within 3 days). This was followed by recovery (by around 7 days), and augmented CTL activity as detected by the hapten-self and not by the allogeneic CTL systems (days 9-13). The injection of F_1 mice with doses of MCMV plus parental spleen cells each of which alone did not drastically reduce CTL potential, resulted in synergistic effect which abrogated CTL potential.

Significance to Biomedical Research and the Program of the Institute: Cyto-megalovirus infection is one of the major problems currently facing human bone marrow transplantation, and may become critical in patients undergoing a chronic GVHR. An understanding of the genetic and mechanistic paramaters involved in resistance and susceptibility to CMV in the murine model, the immunosuppression associated with CMV infection, and the possible synergistic effects of CMV infection and chronic GVH should be valuable for both basic and clinical purposes.

Proposed Course of Project: A number of inbred, recombinant and F_1 hybrid mouse strains will be studied for their ability to be resistant or suscep-tible to immunosuppression rsulting from MCMV infection. We shall also investigate the genetic and mechanistic aspects associated with the syner-gistic effects of MCMV and the GVHR on immunosuppression.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05100-01 I

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
The Role of HLA Genes in Human Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Shaw	Senior Investigator	I NCI
Other:	R. Hall	Clinical Associate	Derm NCI
	S. Katz	Chief, Dermatology Branch	Derm NCI
	H. McFarland	Asst. Chief, Neuroimmunology Br.	NI NINCDS

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have recently defined a new HLA gene which maps centromeric to the other known genes of the HLA complex. Studies have now been initiated to study the distribution of SB antigens in two different disease populations in order to further understand the role of HLA genes in determining disease susceptibility--multiple sclerosis and dermatitis herpetiformis. In the 38 unrelated MS patients studied, there was no significant deviation in the frequency of SB antigens, relative to 200 normal control donors. However, among the 41 DH patients studied, there was a significant deviation from the normal donors, particularly with respect to an elevation of the frequency of the SB1 antigen and a decrease in the frequency of the SB2 antigen. These data cannot be explained by the known strong association between DH and DR3; rather they indicate that there is an interaction between the HLA-SB and the HLA-DR phenotype in determining the risk for dermatitis herpetiformis.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Previous studies from many laboratories have demonstrated that there are associations between many specific diseases and particular HLA antigens. For virtually all of these associations it is not known: 1) exactly what gene product is involved in the disease pathogenesis (i.e., whether the HLA gene product identified is involved in the disease or some unknown allele with which it is in linkage disequilibrium); and 2) what the mechanism is for the association. The extraordinary number of HLA associated diseases and the overall importance of the HLA region in immune responses suggest that an understanding of these associations may be of rather general relevance.

As we develop new markers of the HLA region (project Z01-CB-05101 I), we expect they will be useful in helping us to map more precisely the gene products involved in these disease associations.

Methods Employed: Patients are selected by diagnostic criteria relevant to the particular disease. Patients peripheral blood lymphocytes are "typed" for SB antigen expression by the primed lymphocyte typing techniques outlined in project Z01-CB-05101 I; they are also serotyped for other HLA antigens under contract N01-CB-04337.

Major Findings: 38 unrelated patients with multiple sclerosis were studied. The distribution of SB antigens in this population did not differ from that in 200 normals. Thus, despite a modest increase in frequency of DR2 (and B7) in MS patients there is no marked alteration of SB antigens. This suggests that the HLA gene product involved is more closely associated with DR than SB.

In contrast, in studies of dermatitis herpetiformis, a striking alteration of SB antigen distribution was observed. The results of typing on the first 12 patients were analyzed retrospectively, hypotheses were formulated, and tested on the next 29. In both groups there was a striking increase in the frequency of SB1 (42% vs. 11% in normals) and decrease in SB2 (12% vs. 31%). These alterations could not be explained by the known strong association of DR3 and DH. Instead they suggested that among individuals with DR3, the risk of DH was increased about 2-3 fold by having the SB1 antigen but decreased about 5 fold by having the SB2 antigen. Thus, there is an interaction between the SB and DR phenotypes in determining the risk for DH.

Significance to Biomedical Research and the Program of the Institute: Many diseases are known to be HLA associated, including certain malignancies. Understanding of the role of HLA genes in the pathogenesis of these diseases might reasonably be expected to help in therapy and prevention of these diseases.

Proposed Course of Project: The information on DH is consistent with two genetic models. First, it is possible that there are two independent HLA-linked "disease control genes": DR3 increases the risk of DH (or an allele of another gene in strong positive linkage disequilibrium); SB2 decreases the risk for DH (or an allele of another gene in strong positive linkage disequilibrium with SB2). Second, it is possible that there is a single "disease control gene"; this disease gene does not occur randomly on HLA haplotypes, but predominantly on DR3-positive, SB2-negative haplotypes. Differentiation of these two hypotheses will be possible with family studies of DH patients.

With the SB marker system it will be informative to examine a number of other diseases which are known to be associated with DR3, such as juvenile onset diabetes mellitus and myasthenia gravis. This should help resolve whether all of these diseases have in common a gene which predisposes to autoimmunity, or whether they have different disease genes, each of which is in positive linkage disequilibrium with DR3.

Publications

Shaw, S., and Shearer, G. M.: Cytotoxic T cell interactions with antigen: potential relevance for drug-related systemic lupus erythematosus. Arthritis Rheum., 1981, in press.

Kaslow, R. A. and Shaw, S.: The role of HLA in infection: A review and perspective. Am. J. Epidemiol., 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05101-01 I

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Definition of Gene Products of the Human Major Histocompatibility Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Shaw	Senior Investigator	I NCI
Other:	G. Shearer	Senior Investigator	I NCI
	W. E. Biddison	Expert	I NCI
	L. Lampson	U. Penn, Phila. PA	
	N. Reinsmoen/M. Segall/F. Bach	U. Minn., Mpls, MN	
	G. Pawalec/P. Wernet	Tubingen, Germany	
	A. Termijtelen/J.J. van Rood	Leiden, Netherlands	
	C. Mawas	Marseille, France	
	P. Kavathas	Stanford U., Stanford CA	
	R. Duquesnoy	Milwaukee, WI	

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8 (NIH)	0.6 (NIH)	0.2 (NIH)

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using two different cell-mediated typing assays (secondary lymphocyte proliferative responses and secondary cell-mediated cytotoxicity) we have defined five new HLA-linked antigens designated "SB antigens" (secondary B cell). A reliable system of primed lymphocyte typing for these antigens has been perfected using frozen reagents, and a statistical algorithm for scoring the results is used. Genetic studies suggest that the SB antigens are encoded by a single HLA-linked gene. These antigens have striking similarities to the DR antigens with respect to genetics, tissue distribution and function. However, studies of recombinant families and of mutant cell lines demonstrate that the SB antigens are encoded by a gene distinct from DR, which maps between HLA-DR and GLO. The discovery of this new gene in our laboratory has prompted a large number of collaborations, both to utilize the expertise of other laboratories, and to help others learn to define the SB gene products.

Project Description

Objectives: Genes of the HLA region are important in tissue transplantation, immune regulation and disease association. Consequently, it is crucial to define the gene products of this region and to determine the function of these gene products. There has been a worldwide effort to do so, primarily by serologic techniques. We believe that T cells may be the most sensitive probe for defining intricacies of the HLA region, since T cells seem to be uniquely committed to recognizing gene products of this region. Therefore we expect refined approaches to cellular typing to allow definition of new HLA gene products. Once appropriate tools have been found to define these antigens, we will investigate the genetics of these markers, the tissue distribution of the markers, and function of the markers in cellular immune responses.

Methods Employed: Human PBL are obtained from donors by phlebotomy or batch leukapheresis; mononuclear cells are separated by density separation, and cryopreserved. Analysis of the serologically defined HLA markers on the donors cells is performed by microcytotoxicity testing under contract NO1-CB-04337. Cells from carefully selected donors are sensitized in vitro in one way mixed lymphocyte culture, and generally the primed cells are restimulated after 10 days with the same stimulator to enhance weak responses. Primed cells are frozen in large batches, and thawed as necessary to provide a standard "reagent". Proliferation of these cells in response to stimulator cells is measured by ^{3}H-thymidine incorporation. Cytotoxic activity is analyzed by short term ^{51}Cr release assays using as targets T lymphoblasts or lymphoblastoid B cell lines. The assignment of specificities to a donor's cells is made on the basis of a statistical technique called centroid cluster analysis, which has been improved and adapted for this application.

Major Findings: Five new histocompatibility antigens, designated secondary B cell (SB) antigens have been identified by secondary allogeneic proliferative and cytotoxic responses. The reagents used to define the SB antigens are lymphocytes primed between donors matched for all previously known HLA antigens. To standardize typing for these antigens, cryopreserved primed lymphocytes are now used as standard reagents, and a technique of cluster analysis has been modified to score typing results objectively. Two primed reagents are used to define each SB antigen; although derived from independent responder-stimulator combinations, the concordance between reagents is good. Using this carefully controlled approach to typing, we have studied the genetics of these SB antigens in populations and families. The SB antigen distribution in a population of 215 normal donors is consistent with Hardy-Weinberg equilibrium of alleles of a single locus. Estimated gene frequencies ranged between 3% (SB5) and 36% (SB4) with approximately 31% blanks. Analysis of association between the SB and HLA-A,B,DR antigens in 200 normal donors revealed that associations were generally weak with a few exceptions, in particular the A1,B8,DR3,SB1 haplotype and also the B7,DR2,SB5 haplotype. The segregation of the SB antigens has been studied in 25 families. In all cases the inheritance is consistent with autosomal dominant inheritance of alleles of a single locus. The SB locus is tightly linked to the other HLA genes, but is separable by recombination. The results of 7 HLA-recombinant families indicates that the SB locus maps between HLA-DR and GLO.

The genetics of the SB locus have also been studied in gamma ray induced HLA-mutant cell lines. The conclusion that HLA-DR and SB are independent genes has been confirmed by the observation that some of these mutant lines have lost HLA-DR, but not the product of the cis-linked SB gene.

The tissue distribution of the SB antigens has been examined by studying different cell types as targets for (and inhibitors of) cell mediated lysis. These studies indicate that the SB antigens are selectively expressed on B cells and macrophages, but weakly, if at all, on T cells.

The structure of the SB antigens cannot be definitely determined until the molecule can be isolated. At present, no alloantisera have been identified which recognize the molecule. However, the best available evidence suggests that SB antigens have a molecular structure similar to HLA-DR--a two chain glycoprotein with 33,000 and 29,000 mw chains. This is inferred from the finding that a monoclonal antibody which brings down only material of that description is able to inhibit some SB-specific CML.

The SB antigens have been studied for their function in allogeneic cellular immune responses. They induce a small primary proliferative and cytotoxic response, but this response is augmented in secondary responses. Of particular interest is the finding that the proliferative and cytotoxic responses to the SB antigens are not "restricted" by the other known HLA antigens; this suggests the SB antigens are "major" histocompatibility antigens not only because they are encoded by a gene in that region, but because they function in cellular immune responses independently of the other HLA antigens.

Similar studies are just beginning in two other areas which may reveal new systems of HLA antigens. One of these is a system of antigens which is recognized by cytotoxic T cells on cells which express the HLA-Bw44 antigen. At present two antigens can be defined. Indirect evidence suggests that the gene encoding these antigens (or closely associated antigens) may control the expression of the Bw44 molecule on platelets (itself an unexplained phenomenon) and may regulate influenza-specific immune responses to HLA-Bw44.

Significance to Biomedical Research and the Program of the Institute: Because genes of the HLA region are crucial in controlling immune responses, transplantation, and increasing the risk of a large variety of diseases, therapeutic intervention related to these phenomenon may depend on further understanding of the genes in this region. The SB gene defined already in this project promises to be a very informative one. Since it maps quite a distance from the other known HLA markers, it will provide an important new marker for population studies. Furthermore, the SB gene product itself may be important in immune regulation and disease. Its similarities to HLA-DR and the murine Ia antigens suggest that it may be involved as an Ir gene (controlling immune responses). Furthermore, initial studies (project Z01-CB-05100 I) suggest that it may be a useful new genetic marker for the disease dermatitis herpetiformis.

Proposed Course of Project: We plan to pursue functional studies of the SB antigens with respect to their role in cellular immune responses to foreign antigens and their possible function as Ir genes (probably under project Z01-CB-05067 I). Furthermore, we expect to be involved in structural studies of the SB molecule by identifying antibodies (monoclonal or conventional) which interact with the SB molecule and collaborate with a laboratory able to do structural studies of the relevant molecule. We will continue studies of the relevance of this new marker system in disease (project Z01-CB-05100-01 I).

This work has attracted considerable attention from the world community of scientists interested in HLA. Of necessity, we will be the world reference laboratory for defining the SB antigens, until other laboratories are prepared to assume this function.

We will also be pursuing this approach in definition of other segregant series of antigens such as the Bw44 related antigens alluded to above.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05102-01 I

PERIOD COVERED

October 1, 1980-September 30, 1981

TITLE OF PROJECT (80 characters or less)

Morphologic Studies of Cellular Interactions in the Immune System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. P. Henkart	Expert	I NCI
Others:	P. A. Henkart	Chemist	I NCI
	P. J. Millard	Biologist	I NCI
	T. T. Timonan	Visiting Associate	LID NCI
	J. R. Ortaldo	Biologist	LID NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.9	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒(b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Interactions of positively identified human natural killer (NK) cells known to be killing appropriate target cells have been studied in serial thin sections with the electron microscope. NK cells have a characteristic type of granule containing bundles of tubules in crystalline arrays. The tubule bundles resemble structures formed by microtubule protein (tubulin) under some conditions. Within an hour of binding to a target cell several types of granule within the killer cell, including tubule-containing granules appear to fuse and the heterogeneous contents of the fused granules are secreted by exocytosis into the space between the killer and target cell. The secreted material is mostly in the form of membrane-like structures some of which have ring-shaped profiles on their surfaces. The morphology suggests that tubulin associated with membrane vesicles is released from the killer cell. The secreted products cling to and possibly fuse with the target cell membrane.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: (1) To study the ultrastructure of interactions between identified cell types involved in various aspects of immune responses and (2) To correlate morphologic observations with functional studies. The first system being approached in this way is the interaction of human natural killer cells with appropriate target cells.

Methods employed: Basic morphologic studies are done using standard techniques of electron microscopy. For studies of particular cellular interactions study of serial thin sections is necessary. Special stains and extracellular tracers are used as required. Immunocytochemistry using fluroescent labelled antibodies for light microscopy and ferritin-labelled antibodies at the EM level are used for identification of cellular constituents.

Cells are obtained from peripheral blood of normal adult human donors. Fractions of lymphocytes from discontinuous Percol gradients are prepared by published techniques (Timonen & Saskela, J. Immunol. Methods 36: 285, 1980). Killer-target conjugates are made by centrifuging together and rediluting the the natural killer cell enriched lymphocyte fraction with NK susceptible targets, the human leukemia cell line, K-562. To ensure that all lymphocytes forming conjugates are active killers, the lymphocytes are treated for 2 hours before conjugate formation with human interferon. Under these conditions virtually all lymphocytes that bind target cells kill them within 4 hours.

Major findings: All positively identified NK cells as well as a high proportion of the cells from the percol gradient enriched for NK activity contain a characteristic type of cytoplasmic organelle which is a membrane enclosed bundle of tubules some of which may be in crystalline arrays. (These tubule bundles had previously been observed by us and shown to be a characteristic feature of human peripheral blood lymphocytes bearing receptors for the Fc portion of the IgG molecule as defined by their adherence to antigen-antibody complexes.) The detailed structure of these organelles and their sensitivity to vinblastine suggested that they contained microtubule protein. NK cells also contain a variety of other granules distinguished on the basis of their morphologies.

In NK cells that have formed conjugates with appropriate targets the contents of granules becomes more heterogeneous, apparently because of fusion of several granule types. In some cases tubule bundles are seen in the same granules as numerous small vesicular structures similar to those contained in classical multivesicular bodies. Material similar in appearance to the heterogeneous granule contents including fragments of tubule bundles and membrane vesicles are also found in the extracellular space between killer and target cell. The membranous extracellular material sometimes bears superimposed ring-like profiles similar in diameter to microtubule cross-sections. The simplest interpretation of the morphologic observations is that the mixed contents of the granules

are secreted by exocytosis. The images seen in the EM suggest further that the membranous material released from the killer cell may fuse with the target cell.

Significance to Biomedical Research and the Program of the Institute: Ultrastructural studies of cellular interactions in the immune system promise to contribute much to the understanding of mechanisms of immune functions. Until recently, however, this has not been profitable approach because of the heterogeneity of cell types involved in immune responses. With the recent development of techniques for isolation, characterization, and, in some cases, cloning of lymphocyte populations with specific functions it has become feasible to study cellular interactions at the ultrastructural level.

Attack of tumor cells by cytotoxic lymphocytes is probably important in normal defense against neoplasms. Understanding of the mechanisms by which cytotoxic effector cells kill their targets is fundamental to the potentially useful ability to manipulate cytoxic lymphocyte function for prevention or therapy of neoplasms.

Proposed Course of Project: Immunocytochemistry with anti-tubulin antibody will be used to determine whether the tuule bundles actually contain tubulin and whether tubulin is secreted and transferred to target cells. We intend to compare the interactions of NK cells and their targets with the interactions of antibody-dependent cytotoxic effectors and cytotoxic immune T cells with their respective targets to determine whether similar mechanisms are involved.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05006-16 I

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Lymphocyte Cell Surface Antigens, Normal and Neoplastic

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Mann	Senior Investigator	I NCI
Other:	C. Murray	Guest Worker	I NCI

COOPERATING UNITS (if any) William Blattner and Mark Green, Epidemiology Branch, NCI S. Broder and A. Muchmore, Metabolism Branch, NCI, B. Haynes, Laboratory of Immunology, NIAID, and David Poplack, Pediatric Oncology Branch, NCI

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Biologic, functional and chemical characterization of cell surface structures of normal lymphoid and malignant cells are under investigation. Antigen (HLA) controlled by the major histocompatibility complex (MHC) have been assessed in diseased populations and at risk families. Results indicate that several interacting genes and gene orientation predispose to disease states and immune response. Immunoprecipitation analysis of antigens controlled by the MHC DR gene region suggest at least 3 gene products. Antisera detecting some of these antigens inhibit antigen stimulation and Ig synthesis when these sera are reacted with monocytes and acute lymphocyte leukemia cells. This inhibition has been determined to be due to suppressor T cell activation. 3 monoclonal antibodies 3A1, 4F2 and 5E9 have been characterized for antigen specificity molecular weight of antigen detected and normal cellular distribution of antigens. These monoclonal antibodies have been used to determine phenotypes of malignant cells of lymphoid origin. Cells from patients with Sezary syndrome are $3A1^{-}$ $4F2^{+}$, a phenotype not found on normal cells. Acute lymphocytic and myeloid leukemia cells exhibit a wide variation in antigen expression suggesting dedifferentiation of cells undergoing malignant transformation.

Project Description

Objectives: The objectives of these studies are as follows: a) To identify genes in the major histocompatibility complex which have an association with disease states; b) to identify potential immune response genes in man; c) to assess the biologic function of different cell surface structures in relationship to immune function of lymphocytes; d) to isolate and purify gene products of the major histocompatibility complex for structural analysis; and e) to identify lymphocyte cell surface markers using monoclonal antibodies which react with functional subsets of lymphocytes.

Methods Employed: Gene products of the major histocompatibility complex are identified by dye exclusion cytotoxicity tests. In order to assess the presence of these antigens, subpopulations of cells were isolated as follows: monocytes are recovered after adherence to a glass surface using an EDTA reagent. The non-adherent cells are then plated on plastic surfaces previously coated with an antiserum to the F(ab) component of Ig. This technique isolates the immunoglobulin bearing B lymphocyte. These cells are removed by competition with human Ig. The remaining cell population is considered the T cell population and is then used for cell surface antigen identification or recombined with the other subpopulations of lymphocytes isolated by the above procedures for biologic study. The frequency of HLA alloantigens in a disease population are compared with frequencies in a normal population and/or related disease population by Chi square analysis. Antigen associations were determined by comparing frequencies of antigens occurring together in the disease population and compared with the association of these antigens in a normal population. Related disease groups were compared in a comparable fashion. In family studies haplotype association with the disease was determined by lode score analysis.

The generation of suppressor cell function using anti-B cell sera and monocytes was performed as follows. Isolated monocytes were exposed to antigen (SKSD, Candida tetanus toxoid) washed and then reacted with the antisera. In other studies, monocytes were treated separately and then incubated with autologous or allogeneic T lymphocytes. Tritiated thymidine incorporation was measured after 5 days. To measure the suppression of Ig synthesis, hetero anti-B cell serum was incubated with peripheral blood lymphocytes and the cultures stimulated with the mitogen pokeweed. Levels of IgG, IgA and IgM in supernatants from these cultures were measured by a radioimmunoassay.

The interaction of the various cell populations in allogeneic mixed lymphocyte reaction employs the standard techniques of lymphocyte culture using tritiated thymidine incorporation as a measure of response. Cell surface antigens have also been investigated employing flow microfluorometry using the fluorescence activated cell sorter. Lymphocytes or subpopulations thereof were isolated as described above for analysis. Monoclonal antisera products of genes of the major histocompatibility complex are incubated with the cell populations, the cells washed and a fluorescinated goat anti-mouse Ig antibody used to identify the reaction of the antibody to the cell surface.

Monoclonal antisera were also directly labeled with fluorescien and incubated with lymphocytes or subpopulations thereof. After extensive washing, the cells are analysed with the fluorescence activated cell sorter.

Studies of the molecular characteristics of human B cell alloantigens were performed by labelling cell membrane components with tritiated amino acids. The cell membrane components were solubilized with non-ionic detergents and purified by gel filtration chromatography and Conavalin A lectin column purification. After elution of the adhered antigen with alpha-methylmannose appropriate anti-DRw (or anti-human Ia antisera) as well as control sera were incubated with the isolated material and the antigen-antibody complexes precipitated with the Cowan strain Staph. aureus organism. The antigen antibody complexes were dissociated with sodium dodecyl sulfate in the presence of the reducing substance mercaptoethanol. These dissociated materials were electrophoresed on polyacrylamide gels containing sodium dodecyl sulfate. Gel slices were counted in a liquid scintillation counter and the migration of the isolated labelled proteins assessed. In some instances sequential precipitations with the anti-DR sera or anti-Ia sera were performed. In other studies, combinations of antisera were used in precipitation analysis.

Major Findings: HLA-B, C and DRw antigen determinations are being performed in a normal population and in a variety of disease conditions. These studies are carried out in part in this laboratory and supported by an NCI contract N01-CB-53890 to Duke University. The major observations are as follows: analysis of HLA alloantigen profiles in patients with rheumatoid arthritis revealed an association of antigenic determinants controlled by multiple genetic loci in the major histocompatibility complex. HLA-A1, B40, DR4 and MT2 antigens were associated in combinations more frequently than in the normal population. The results suggest the hypothesis that multiple genes within the MHC predispose to the disease state. Studies in several other disease conditions reinforce this hypothesis. Three families with multiple cases of primary and secondary Sjogren's syndrome were studied. The MT1 and MT2 antigens were found in trans haplotype position in only those affected family members with the primary disease. In those individuals with secondary Sjogren's syndrome (rheumatoid arthritis) the MT1 antigen was not present and DR4 and MT2 were found on the trans haplotypes. Patients with psoriatic arthritis were classified as to disease severity. HLA alloantigens found to be increased in frequency in the total diseased population (HLA-A26, Bw38, Cw6, DR7, Ia744) were found to have different associations depending on severity of disease. These results again suggest interaction of multiple MHC genes in disease predisposition. Association of HLA alloantigens were also found in studies of insulin allergic diabetes. The frequency of combinations of HLA-A2, B44, DR7 occurred in 39% of insulin allergic diabetics, 5% in nonallergic diabetics and 32% of normal. The results indicate that gene(s) linked to the genes controlling the expression of the above antigen predispose to allergic reaction in diabetics receiving insulin.

The role of the monocytes in a mixed lymphocyte reaction was studied. The presence of monocytes was demonstrated to be required in either the stimulating or responding cell population in order for stimulation to be observed. In these studies antisera directed to the DR antigens on the monocytes of the responding population were observed to inhibit the MLR. This inhibition was demonstrated to be due to the induction of suppression by these monocytes in the autologous cell population.

These studies have been extended to investigate the mechanism of suppression. Both allo and hetero anti-DR sera inhibit antigen stimulated lymphocyte cultures and pokeweed induced in vitro immunoglobulin synthesis. This inhibition was found to be due to induction of suppressor T cell function based on the following observations. T cells were isolated from lymphocytes cultured with monocytes exposed to anti-DR sera for 24 hours. These T cells effected suppression of Ig synthesis and antigen stimulated lymphocytes. Low doses of irradiation (2000R) in primary cultures reversed the suppression. $F(Ab')_2$ fragments of hetero and allo anti DR sera did not induce suppression. In experiments designed to negate the possibility that anti-DR sera were simple blocking antigen presentation by monocytes, acute lymphocytic leukemia cells were reacted with anti-DR sera and added to autologous lymphocytes. These antisera treated cells were capable of suppression induction. Sera from patients with ALL (active disease and remission) were capable of induction of suppression when reacted with ALL cells.

Monoclonal antibody was produced by the immunization of BALB/c mice with the human HSB-2-lymphoid T cell line. The spleen cells were fused with the P3X63/Ag8 BALB/c myeloma cell line. Antibody from these cell lines have been isolated by ammonium sulfate precipitation and isoelectric focusing. The antibody was labeled with fluorescein or tested in a sandwich technique using goat anti mouse k chain reagent. Six antibodies have been well characterized. 3A1 antibody reacts with 85% of peripheral blood T lymphocytes and precipitates a 40,000 dalton molecular weight molecule from the T cell membrane. The 4F2 antibody reacts with monocytes and thymocytes and with less than 5% of the T cells and precipitates an 80,000 dalton component. The 5E9 antibody detects an antigen on thymocytes and activated T cell population. Three antibodies precipitate molecules of 44,000 and 12,000 daltons and are thought to react with HLA.A, B and C like antigens in that these sera react with all peripheral blood lymphocytes. However cold inhibition studies with these antibodies demonstrate lack of cross blocking of antibody indicating that they are not reacting with the same determinants on the molecule.

These monoclonal antisera were used to study a patients population with Sezary syndrome. Cells from these patients were $3A1^-$, $4F2^+$ Ia^+ thus demonstrating a distinct cell surface antigen phenotype in this malignancy. Cell surface

antigens were also determined on cells from patients with acute lymphocytic (ALL) and myelocytic leukemia (AML). using the above monoclonal antibodies and in addition the OKT3, OKT4, OKT6, OKT8 monoclonal antiserum. The 3A1 antigen was found in relatively high density on T cell ALLs and was not present in appreciable amounts on non T, non B cell ALLs. The patterns of reactivity of the other monoclonal antibodies with ALL cells varied from patient to patient. No constant pattern of combinations of antigens was observed. The reported observation that the patterns of antigen distribution found in normal thymocytes during differentiation could also be found in ALL were not confirmed in this study. The presence of antigens, specific for normal T cells, on non T, non B cell leukemias and on cells from patients with AML supports the hypothesis of cellular differentiation in ALL and AML rather than a malignant clonal expansion of normal cells.

Immunoprecipitation studies with alloantisera detecting B cell alloantigens demonstrate a complex series of molecular components depending on the cell of origin and antisera used. Hetero anti-B cell sera precipitate detergent solubilized components having molecular weights of 34,000, 31,000, 28,000 and 27,000 daltons. Alloantisera detecting different DR MT and MB precipitate combinations consisting of 2 or 3 of the above molecules. The results are consistant with the interpretation that multiple genes control the expression of the B lymphocyte alloantigens.

Significance to Biomedical Research and Program of the Institute: Human B lymphocyte alloantigens appear to be analogous to the B cell alloantigenic system in mice. Genes controlling these antigens in mice appear to be linked to or associated with the ability of the animal to respond to particular antigens. Identification of human alloantigens together with a description of the fine structure of the genetic complex is necessary for the understanding of the relationship of the MHC to disease states. Our studies of the various disease entities described above suggest that multiple genes in the MHC are associated with the disease entity or a manifestation of the disease. Studies in the diabetic population where specific allergic manifestations to insulin or insulin components have been identified, strongly indicate the presence of a specific immune response gene in man.

Monocytes express DR alloantigens and appear important in the initiation of the immune response. The mechanism of communication of the monocyte the T and/or B cells in activation of immune response is unknown. The demonstration that an intact antibody to HLA-DR antigen induces a strong signal for induction of suppressor cells establishes a new concept in immunology. The biologic relevance of this observation relates to a model for control of immune response by a biofeedback mechanism. These observations tend to support the hypothesis that the molecule structures coded for by genes that determine DR antigens serve a role in cell-cell interaction. The observation that leukemia cells bear DR or DR like determinants and that sera from these patients induce immunologic suppression may explain the lack of a normal response to cells undergoing neoplastic change. Since these DR determinants appear to be

different from those found on normal cells, production of antibody to the DR determinants on neoplastic cells may lead to suppression of immune response to the malignant cells. This hypothesis is supported by the observation that a variety of neoplastic cells bear DR like antigens and our observation that sera from patients with leukemia induce suppression when reacted with leukemic cells.

Monoclonal antibodies to determinants expressed differentially on lymphocytes are potential tools in isolation of functional subsets of lymphocytes and the study of interaction of lymphocyte populations in the immune response. Once characterized, these antisera may be used in the identification of alteration of lymphocyte subpopulations in the disease states. One antibody under study has demonstrated reactivity against peripheral blood cells of patients with Sezary syndrome and not reactive with T cells from normal individuals. These sera can thus potentially be used to identify malignant cells for diagnostic or therapeutic procedures.

Studies of molecular components reacting with antisera detecting B cell and monocyte antigens gives insight into the possible role of these cell membrane structures in immune response. Our observation that alloantisera reacting with these antigens precipitates different size molecules depending on the cell source suggest that recombination of several molecules on the cell surface can occur. Such rearrangements may have important influences on cell-cell interactions. Our observations that the DR or Ia like antigens have different associations in disease states than in the normal population suggest that molecular rearrangement of these structures on cell surfaces may predispose to disease condition.

Proposed Course of Project: Studies in the various disease entities mentioned above will be continued. The studies are now and will be directed primarily to families particularly those wherein more than one member has a malignant disease condition. The goal is to determine the potential for cooperative gene effects in the role of the pathogenesis of the disease. Families in which there are multiple cases of malignancies will be HLA typed and studied for in vitro immune response capability. In addition lymphocytes from the family members will be studied for cell surface markers using monoclonal antibodies that are reported to differentiate functional subsets of lymphocytes. Information obtained from this multidirectional approach will be used to attempt to determine the role of the MHC and other genes in immune responsiveness and to attempt to define immune alteration in patients with malignancies. These studies will be performed in collaboration with the family studies section of the Epidemiology Branch, NCI.

Studies will continue in attempts to define specific biologic function of the molecular structures bearing the human DR antigens. These studies will be directed towards understanding the role of these structures in activating or suppressing immune response. These studies will be extended to patients with malignancies to amplify our observations that sera from acute leukemia patients contain substances (non-cytotoxic antibodies?) that induce suppression when reacted with leukemia cells.

Further characterization of the molecular structure of HLA-DR antigens will be undertaken. The specific goals will be to isolate and chemically characterize the molecules that bear the different antigenic determinants.

Publications

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SUMMARY REPORT

MACROMOLECULAR BIOLOGY SECTION, IMMUNOLOGY PROGRAM
DCBD, NCI

October 1980 - September 1981

The Macromolecular Biology Section is a small unit in the Immunology Program, reporting directly to the Director, DCBD, NCI. The common interest of the investigators is the elucidation of macromolecular changes on the surface of mammalian cells, and how such changes relate to certain normal (differentiated) cell surface functions pertinent to cell division and to the appearance of cellular tumorigenicity.

Sid Shifrin developed a very productive cooperative study with investigators centered around Dr. L. D. Kohn in the LBP at NIAMDD, on the binding of thyroglobulin to the thyroid receptor in the bovine thyroid plasma membrane. Ingenious chemical modifications of either of the lysyl or of the tyrosine residues of the thyroglobulin, devised by Sid Shifrin, showed that the site of thyroglobulin iodination on the tyrosine residues is the important determinant in the receptor binding process. Another approach, in which the carbohydrate chain of the thyroglobulin was altered by removing enzymatically either sialic acid or galactose, which resulted in the appearance of an altered higher sedimenting form of the thyroglobulin, demonstrated that the recognition of N-acetylglucosamine residues and the extent of iodination in the altered thyroglobulin is also involved in the binding to the specific receptor site. There are large numbers of thyroid tumors which affect the structure of the thyroglobulin through it its biosynthesis, and also affect its biodegradation. Thus the elucidation of the nature of the binding site on the thyroid membrane and the structural features of the thyroglobulin required for binding and degradation has importance to the program of the NCI.

The changes which occur in proteins and in other biologic macromolecules after SV40 induced or after spontaneously occurring transformations in tissue culture of mouse and other mammalian cells were studied. Dr. Chandrasekaran, together with Mrs. V. W. McFarland found that an ~55,000 MW cellular protein which is "induced" after SV40 infection of cultured mammalian cells, is also present in embryo primary cells which were not exposed to the SV40 virus. The mouse embryo protein and the "induced" protein in SV40 transformed mouse cells when labelled with ^{35}S-methionine have virtually similar 2D tryptic peptide fingerprints. The monoclonal antibody to the SV40 induced protein precipitates the embryo protein. Both types of proteins are phosphorylated mainly in the serine residues both by $^{32}PO_4$ *in vivo* and also in the immunoprecipitate by ^{32}P ATP. The latter result implies associated specific kinase activity, also observed by other investigators. Mouse, rat and hamster embryo ^{35}S-labelled 55K proteins and the 55K SV40 induced proteins from the same species are structurally identical, but have species divergence approximating the expected evolutionary order and divergence. Portion of the 55K SV40 induced protein was found by biochemical techniques on the cell surface (work with Dr. Luborsky). Immunologic crossreactivities imply that it also may be on the surface of the embryo cells.

All of these make it likely that the 55K protein has (or had) an important, i.e. evolutionary conserved, cellular function. We do not know what this function is, or whether it is important in embryonic differentiation. However, Dr. Chandrasekaran and Mrs. McFarland found a significant decrease during the development of the embryo: the amount of the 55K protein in the primary cells or in the organ culture from 12 day old mouse embryos is high, but decreases to a very low level from 16 day old mouse embryos. Replating midgestation embryo primary cells from mouse, rat and hamster leads to a great decrease in the 55K protein.

Thus, the established cells lines or clones, which are mainly fibroblasts, have only very low amount of the protein, be they normal cells or highly tumorigenic spontaneously transformed (sarcomagenic) cells. Dr. Chandrasekaran found that in both the spontaneously transformed cells and in normal cells, the half life of the protein is very low ($\leq$60 min.). In SV40 transformed cells the SV40 coded T antigen appears to stabilize the 55K protein by direct interaction. However, he cannot find signs of any interacting proteins in those cells which are not transformed by SV40 (embryo primaries, mouse embryonal carcinoma cells, L cells and neuroblastoma cells) but contain substantial amounts of the 55K protein. Dr. Chandrasekaran and Mrs. McFarland could not find any correlation of the amounts of the cellular 55K protein with the cell division rate, or a decrease in the amounts after contact inhibition of cell division in established cells.

Nevertheless the 55K protein may provide a unique activity for study. This protein together with the SV40 T antigen, appears on the surface of the SV40 transformed cells, as well as in their nuclei (Drs. Luborsky and Chandrasekaran). Thus the 55K cellular protein may represent as it does a "contact point" reaching back to the DNA level in the regulatory pathways in DNA replication and cell division in two systems: the differentiating embryo cells and the SV40 transformed cells. Dr. Luborsky recognized this, and initiated a careful study on the interaction of the 55K protein with DNA, similar to the interaction of the SV40 T antigen with (calf thymus) DNA originally found by Dulbecco. A fraction of the SV40 T antigen and also of the 55K protein, isolated from nuclei or from surface membranes of SV40 transformed cells, were found indeed to interact with calf thymus DNA, as eluted only at elevated pH and salt concentrations. Similar fractions of the 55K proteins are now being obtained from the mouse embryonal carcinoma F9 cells which contain the 55K protein but not the SV40 T antigen. The latter result implies that the 55K protein (from both the nuclei or from the surface membrane) indeed interacts with the cellular DNA directly, not just because through the SV40 T antigen with which it is in complex in the SV40 transformed cells. Dr. Luborsky plans to expand this work to study the midgestation embryo cells. These interactions may open up novel studies on the effect of new type of proteins in regulating cucaryotic cell growth and differentiation.

Much of the work on the 55K proteins in other laboratories is on transformed or tumorigenic cell lines. We do not find a general correlation with tumorigenicity when the spontaneously occurring tumorigenic transformed mouse fibroblast cells (clones) are compared with the normal clonal parent cells, in very carefully matched cell families. We feel that the importance of our discovery is in the finding that the 55K protein is being expressed constitutively in normal embryogenesis, and in the finding that the same cellular gene expression being modulated in many transformation processes such as in SV40 transformation. This can lead to the convergence of two fields of investigations: There is now a potential avenue of applying molecular biology techniques and reagents which are so well defined in tumor virology, to some yet undefined changes which occur in

in embryogenesis.

There was considerable research use of the well defined mouse cell families developed painstakingly for genetic and biochemical studies during the last several years (Mora and McFarland). Dr. Winterbourne, who carried out a very careful study on the glycosoaminoglycans on the surface of cells, found that both spontaneous transformations and the SV40 transformations of the same parent clonal mouse cell, which are both very rare events, result in a highly specific change in the biosynthetic pathways: the reduction in the 6-O-sulfate glucosamine in a particular region of the heparan sulfate molecules. Careful analysis of our genetic and biologic data with his biochemical findings led us to conclude that this common biochemical change could not just happen by "chance", it may have a common correlate in the increase in the synthesis of cellular DNA accompanied by increased activities of the appropriate enzymes in both spontaneously occurring and by SV40 induced cellular transformations. Dr. Winterbourne returned to England, but continues these studies on our cells.

We also have an unparalleled collection of spontaneously transformed clonal mouse cells in closed families with careful control on the pedigree and on the selection factors both in vivo and in vitro, (clones obtained with V. McFarland and L. Waters). We think this allows us to set up a unique and rational approach for the analysis of the seemingly intractable problem of "spontaneous" (chance) transformation. One interesting biologic finding shows that the highly tumorigenic (sarcomagenic) clonal mouse cells which attained a stable heritable tumorigenic phenotype in a low frequency ($1/10^6$) apparently by a mutational event, as a rule do not grow without anchorage in a viscous medium in vitro. Thus while cells which grow well without anchorage are generally tumorigenic, the opposite is not true. Obviously, for in vivo tumorigenicity (either in the syngeneic or in the nude mouse) further factors are required in the in vivo interaction of these cells with the host, which do show up in the anchorage independent in vitro cell growth experiments. Our cells therefore appear also useful to study such in vivo factors, both in the syngeneic and the nude mouse.

In further characterization of the well pedigreed families of normal mouse cells, or cells transformed spontaneously or with SV40, we find that spontaneous transformation (cf. by somatic mutation) is sufficient to explain the acquisition of cellular tumorigenicity of the SV40 transformed mouse fibroblasts. There is no binding correlation between cell growth in viscous medium, of tumorigenicity in vivo in both syngeneic and in nude mouse and of the SV40 expression in the mouse fibroblast cell families we have investigated. This confirms our previous observation that on the balance the phenotypic changes pertaining to in vivo properties of cells attributed to SV40 early gene coded T antigen are predominantly expressed as cell surface antigens, facilitating immunologic recognition and rejection of the cells in the immunologically competent syngeneic mouse. Our results underline the growing recognition that for the acquisition of tumorigenic potential the transformation by SV40 is not a sufficient cause, and may not even be a (crucial) contributory event in the mouse (or rat) cells. Beside the above mentioned work and that of Dr. Winterbourne, we are exploiting our collection of clonal cells in numerous collaborative studies in this Country and abroad (see Section II and III of Z01 CB 05526-13).

We plan now to investigate the cellular DNA changes in the spontaneous transformations by transfection of normal parent clones with restriction endonuclease treated DNA from the derivative tumor clones.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05097-02 IP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure of Thyroglobulin and Interaction with Membrane Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	L. D. Kohn	Medical Officer	LBP	NIAMDD
	J. E. Rall	Director		NIAMDD
	Paolo Vitti	Visiting Scientist		NIAMDD
	Christian B. Anfinsen	Chemist	LCB	NIAMDD
	Mark Smith	Chemist	LCB	NIAMDD
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COOPERATING UNITS (if any) Frank Maley, N.Y. State Dept. of Health, Albany, N.Y.; William Valente, University of Maryland; Eduardo Consiglio, Salvatore Aloj, Gaetano Salvatore, Paolo Lacetti, University of Naples; Roy Sundick, Wayne State University

LAB/BRANCH
Immunology Program

SECTION
Macromolecular Biology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☑ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to examine the structures of thyroglobulin, thyrotropin (TSH), interferon, and cholera toxins in order to elucidate the structures which are necessary for interaction with receptors on thyroid membranes.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The purpose of these studies is to determine the chemical nature of the structures of several thyroid hormones, toxins and interferon which participate in binding, internalization and biodegradation by thyroid cells.

Methods Employed: Affinity chromatography, two dimensional gel electrophoresis, chemical modification of proteins and receptors, chemical modification and isolation of glycopeptides from glycoprotein hormones, preparation and characterization of monoclonal antibodies, purification of enzymes involved in ADP-ribosylation, ultraviolet absorption spectroscopy, fluorescence, circular dichroism, light scattering, radioautography.

Major Findings: I. Bovine thyroglobulin binds to the thyroglobulin receptor via tyrosyl residues since chemical modification of tyrosyl residues either by nitration with tetranitromethane or by O-acetylation with N-acetylimidazole markedly reduces binding of 19S thyroglobulin to bovine thyroid membranes. 27S thyroglobulin can be prepared in vitro by removing sialic acid and galactose from the B carbohydrate chain of 19S thyroglobulin using neuraminidase and β-galactosidase, respectively. This transformation from 19S to 27S is quantitative if N-acetylglucosamine is also removed from the B chain by N-acetylglucosaminidase. The transformation is facilitated with thyroglobulin containing a high iodine content. The 27S material prepared in vitro has many of the properties also found with 27S iodoprotein made in vivo. The fluorescence is quenched probably because of a nearby iodotyrosine; the lysyl residues are more reactive with trinitrobenzyne sulfonic acid. The 27S thyroglobulin is more readily dissociated by urea and by succinylation than 19S thyroglobulin. The 27S material binds to thyroid membranes to a greater extent than 19S thyroglobulin. Chemical modification of the tyrosyl residues of 27S material with either tetranitromethane or N-acetylimidazole does not interfere with binding to thyroid membranes. The 27S material binds to a different site than 19S thyroglobulin. The 27S material also inhibits binding of TSH. (With Kohn (Z01 AM-23960 LBP, NIAMDD), Rall, Consiglio, Salvatore, Sundick.)

II. TSH inhibits the binding of interferon to functional thyroid cells in culture (FRT_L) and less so to non-functional cells (FRT). There appears to be some common sequences in the structures of TSH, cholera toxin and interferon. Synthetic interferon (22-166) prepared by Drs. Mark Smith and Christian B. Anfinsen binds to functional thyroid cells in culture.

a) Interferon - IF - FRT_L cells have been found to be exquisitely sensitive to the presence of interferon. The cells respond to the interferon within one hour after the addition of the antiviral agent and produce 3',5'-oligo A synthetase. We have found that the preincubation of the cells with TSH prevents the action of interferon on FRT_L cells but has a smaller effect on FRT (non-functional) cells. (With Friedman, Smith and Anfinsen (Z01 AM-25000 LCB, NIAMDD.)

Smith and Anfinsen have chemically synthesized human IF and some variations of human interferon in which phenylalanine has been substituted for tryptophan. These peptides have been radiolabelled by a variety of techniques and their ability to bind to FRT_L membranes has been examined. One of the larger synthetic fragments has been found to bind to a significant extent to these

membranes and the potential biological function of the material is being investigated.

III. TSH Stimulated ADP-ribosyl Transferase in Thyroid Membranes. A variety of TSH derivatives have been prepared in order to examine the nature of the functional groups of this hormone which are operative in this enzymatic reaction. Also cholera toxin derivatives are being examined.

a) Thyroglobulin isolated from chickens which are hyperthyroid or have autoimmune thyroiditis differ in the distribution of 12S, 19S and 27S forms. Thyroglobulin isolated from the OS chickens which have autoimmune thyroiditis is predominantly 12S and binds very poorly to thyroid membranes. Thyroglobulin isolated from hyperthyroid (CS) chickens is predominantly 19S although this form is unstable and rapidly degrades to 12S which binds poorly. Normal chicken thyroglobulin contains 12S and 19S forms. We are examining the reasons for the presence of particular forms in these genetic strains. (With Kohn (Z01 AM-23960 LBP, NIAMDD) and Sundick).

b) Chemical Modification of the Amino Acid Side Chains. Bovine thyroglobulin has been modified by succinylation, trinitrophenylation, nitration and O-acetylation in a study of the effect of these modifications on the structure of the thyroglobulin and on the binding to receptors.

Low degrees of succinylation do not affect the state of aggregation of 19S thyroglobulin but increase binding to thyroid membranes. As the extent of succinylation increases the 19S from dissociates into 12S subunits and the binding to thyroid membranes increases.

Trinitrophenylation of lysyl residues does not affect subunit interactions but does increase binding to thyroid membranes.

Chemical modification of tyrosyl residues either by nitration with tetranitromethane (TNM) or by O-acetylation using N-acetylimidazole did not produce dissociation of the 19S from but markedly reduced binding to the thyroid membrane. Removal of the O-acetyl group with hydroxylanine allowed ^{125}I-thyroglobulin to bind to the membrane.

c) Modification of the Oligosaccharide Moiety. Removal of the terminal sialic acid residue with neuraminidase does not affect the state of aggregation of 19S Tg. However, when the penultimate galactose residue is removed with β-galactosidase from Aspergillus, nearly 30% of the protein has aggregated to the 27S form. This 27S material prepared in vitro has many of the physicochemical properties found with 27S iodoprotein made by the thyroid gland in vivo. For example, the fluorescence of thyroglobulin (19S) is quenched as a result of the association, the emission maximum indicates that the tryptophan residues are in a more hydrophilic environment.

The 27S material prepared either way binds to a much greater extent to thyroid membranes than does the 19S form. While succinylation or trinitrophenylation markedly enhances binding to thyroid membranes, modification of the tyrosyl residues has no effect on binding.

The 19S Tg and 27S form bind to different binding sites on the membrane. The rate of internalization of 19S versus 27S by FRT (non-functional) and FRT_L (functional) cell is currently under investigation.

The removal of the third sugar in the B chain of thyroglobulin (N-acetylglucosamine) by the action of β-N-acetylglucosaminidase from Aspergillus caused more 19S thyroglobulin to aggregate to the 27S form than had occurred after removal of the penultimate galactose residue.

The role of the sugar moieties in binding to thyroid membranes is being investigated in collaboration with Dr. Frank Maley who is isolating the sugar moieties by the use of Endoglycosidase H. Independently, we digested the polypeptide backbone of 19S thyroglobulin with proteolytic enzymes and obtained a crude mixture of the oligosaccharides and some oligopeptides. This crude mixture inhibits binding of ^{125}I-thyroglobulin to thyroid membranes. Upon further separation of the A and B chains over a Dowex column, the inhibitory fragment has been lost.

d) Presence of Phosphomannose. We observed that either mannose-1-phosphate or mannose-6-phosphate can inhibit the binding of Tg to thyroid membranes at pH 5 in the absence of added calcium. Similarly monophosphopentamannose is especially effective as an inhibitor. These results suggested that binding to the membranes may occur via a mannose phosphate residue on the thyroglobulin molecule. All attempts to detect mannose phosphate directly by TFA hydrolysis followed by fluorometric determination with glucose-6-phosphate dihydro ginase, phosphoglucosisomerase and phosphomannoisomerase have failed. However, by using radiolabelled sugar precursors together with ^{32}P, it is now clear that thyroglobulin contains a diester of N-acetylglucosamine and mannose. Whether this grouping is part of the binding iste is not clear. However, the role of this functional group in directing thyroglobulin to the lysosome where it will be degraded to produce the thyroid hormones, triiodothyronine and thyroxine, is under investigation. (With Kohn (Z01 AM-23960 LBP, NIAMDD), Salvatore and Consiglio).

e) Attempts to Isolate the TSH Receptor. Yavin and Valente have prepared monoclonal antibodies against the TSH receptor which will inhibit the binding of ^{125}I-TSH to thyroid membranes. This monoclonal antibody will also activate adenylate cyclase in thyroid membranes. An attempt is being made to prepare an affinity column with the monoclonal antibody, extract thyroid membranes with non-ionic detergents (Triton-X) and isolate the receptor in a manner similar to that described by Ashwell for the hepatic asialoglycoprotein receptor. (With Yavin, Valente, Lacetti and Kohn (Z01 AM-23960 LBP, NIAMDD)).

f) Uptake of Tg and Modified Tg's by Fibroblasts. 19S thyroglobulin and 27S thyroglobulin have been labelled with rhodamine in an attempt to follow their route of uptake and internalization in fibroblasts. Thyroglobulin which was modified with monophosphopentamannose was found to inhibit the uptake of L-iduronidase (Rachel Meyerowitz, NIAMDD) which is taken up via the mannose phosphate receptor in fibroblasts. (With Pastan and Willingham (Z01 CB-08712 LMB, NCI).

Normal human fibroblasts did not appear to have sufficient receptors specific for this sugar phosphate and other types are under investigation -

particularly Chinese hamster cells.

g) ADP-Ribosyltransferase. Cholera toxin possesses ADP-ribosyltransferase activity and although TSH does not have this activity, it can stimulate the ADP-ribosyltransferase of thyroid membranes. There is evidence (Vitti and Kohn (Z01 AM-23960 LBP, NIAMDD) that TSH itself is ADP-robylated in addition to the ribosylation of some membrane compoents including the G regulatory protein.

I have prepared a large number of derivatives of TSH and of cholera toxin in an attempt to determine the ADP-ribosyltransferase site on cholera toxin and to determine the site of ADP-ribosylation on TSH.

Significance to Biomedical Research and the Program of the Institute: There are a large number of thyroid tumors which affect the structure of thyroglobulin, i.e., its biosynthesis as well as its biodegradation. Our studies are directed at elucidating the nature of the binding site on thyroid membranes and the structural features of Tg required for binding and degradation.

Proposed Course of the Project: Monoclonal antibodies against TSH have been prepared and we plan to use it in the isolation of the TSH receptor. Bovine thyroglobulin is being cleaved by enzymatic and by chemical methods in an attempt to determine that portion of the molecules which is involved in binding to the thyroid membrane receptor. We are also purifying ADP-ribosyltransferase from thyroids in an effort to determine how this enzyme provides a mechanism for control of cellular processes.

Publications:

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Consiglio, E., Shifrin, S., Yavin, Z., Ambesi-Impiombato, F. S., Rall, J. E., Salvatore, G., and Kohn, L. D.: Thyroglobulin interactions with thyroid membranes. Relationship between receptor recognition of N-acetylglucosamine residues and the iodine content of thyroglobulin preparations. J. Biol. Chem., 1981, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB-05545-01 IP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Expression of Cellular Antigens in Transformed Cells and in Embryonic Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	V. W. McFarland	Chemist	IP	NCI
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LAB/BRANCH
Immunology Program

SECTION
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TOTAL MANYEARS: 2.5	PROFESSIONAL: 1.5	OTHER: 1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to further identify, isolate and characterize an SV40 induced 55,000 MW cellular protein in embryonic cells. Primary cells prepared from midgestation mouse, rat and hamster embryos expressed the 55,000 dalton protein constitutively without SV40 infection. The 2D tryptic peptide map showed that this protein is similar to SV40 induced 55K protein and is conserved evolutionarily in both embryonic and in SV40 transformed cells. The embryo protein is a phosphoprotein. The amount of the protein in embryo was found to be half that of SV40 transformed cells and its presence correlated with the age of the embryo (in the case of mouse), maximum being at 12 day and minimum at 16 day. The amount of the protein decreased also with the tissue culture passage of embryo primary cells. The expression of the protein did not correlate with tumorigenic transformation in established mouse cells.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To determine whether the SV40 induced 55K cellular protein is expressed in embryonic cells and in spontaneously transformed tumorigenic cells and also to study the location of 55K protein in SV40 transformed mouse cells.

Methods Employed: Preparation of primary, secondary and tertiary cultures from embryos of different periods of gestation. Radioactive labelling, immunoprecipitation gel electrophoresis, electrofocussing, thin layer chromatography and fluorography. Cell growth dynamics in culture.

Major Findings: A specific 55,000 dalton protein was shown to be present in SV40 transformed cells in addition to SV40 coded large T and small t antigens. This 55K protein is not encoded by SV40 DNA but it is of cellular origin. Studies on tryptic peptides revealed that this is an evolutionarily conserved protein. The same or similar protein was detected in a variety of transformed cells induced by various agents such as meth A, x-ray and RNA tumor virus. The same or similar protein was also detected in embryonal carcinoma cell without SV40 infection. The objective of our study was then to find out whether the expression of this protein correlates with embryonic development and/or with tumorigenic transformation. The location of this protein in SV40 transformed cells was also studied, to find some clue pertaining to its possible function.

A) 1. 55K Protein is an Embryo Protein. Mouse embryos at various post implantation stages (10-16 days) were investigated and compared. Primary cultures from 10 and 12 day old mouse embryo showed the presence of specifically immunoprecipitable 55K protein. The presence of this protein was further confirmed by

a. immunoprecipitation of 55K protein from primary embryo cultures with a monoclonal antiserum prepared against SV40 induced 55K protein,

b. the comparison of ^{35}S-methionine labelled tryptic peptides of embryo 55K and SV40 induced 55K proteins indicated similar tryptic peptide pattern (in collaboration with Dr. D. Simmons),

c. the embryo 55K protein was labelled with ^{32}P indicating the protein to be a phosphoprotein similar to SV40 induced 55K.

2. Correlation of 55K with Embryo Age and Tissue Culture Transfer. The presence and the amount of the 55K protein was then investigated and quantitated from different day embryos. The amounts were then compared with the amount of SV40 induced 55K protein. The amount of the protein present in 12 day old embryo primary cells was found to be about 50% compared to SV40 induced 55K. The amount of the protein in older embryos decreased with the age of the embryo, as shown - 13 day - 40%, 14 day - 25%, 15 day - 7.1% and 16 day - 2.6%. Thus the expression of the protein correlated with the age of the embryo.

Attempts were made to identify the organs which are responsible for the synthesis of this protein in embryos. In collaboration with Dr. M. Dziadek we dissected and labelled various organs from 12 day and 16 day mouse embryos. The results showed that in 12 day old mouse embryo the following organs were found to possess the 55K protein - liver, brain, heart, carcass and possibly visceral yolk sac whereas the same organs isolated from 16 day old mouse embryo did not possess the protein. The expression of this protein was studied upon

tissue culture transfer (t.c.t.) of the embryo primary cells. The 12 day mouse embryo primary cells which possessed the protein when trypsinized and replated were found not to contain the protein (the first t.c.t.). Similar results were obtained during the second t.c.t. also. Thus there could be a tissue culture selection against these cells which synthesize the 55K protein.

3. Presence of 55K Protein in Murine Embryos. The presence of the protein in embryos was further confirmed by studying the embryos of other species, i.e. rat and hamster. Immunoprecipitation with the SV40 T serum and with monoclonal serum indicated the presence of 55K protein in 14 day old rat and 10 day old hamster embryo primary cells. The amount of the protein precipitated was found to be 60% in hamster embryos and 45% in rat embryos compared to SV40 induced 55K protein. The first tissue culture transfer cells derived from hamster embryo primary cells synthesized about 10% of 55K whereas the second t.c.t. cells did not show the presence of 55K. On the other hand the first t.c.t. cells from rat embryo primary cells did not show the protein. Thus the hamster embryo primary cells which synthesize maximum amounts seem to retain some cells after first t.c.t. and therefore continue to show the presence of 55K protein whereas other embryo primaries did not. The presence in other embryos is further confirmed by 2D tryptic peptide map. The 2D map of hamster embryo is found to be similar to that of 55K from SV40 transformed hamster cells. Thus the embryo 55K protein also seems to be evolutionarily conserved as is the SV40 induced 55K.

4. Correlation of 55K Protein with Differentiation of EC Cells. Embryonic carcinoma cells (F9 and OTT6050) were tested and found to express the 55K protein. The methionine containing tryptic peptides was found to be similar to SV40 induced 55K protein. The EC cells were then subjected to differentiation by treatment with retinoic acid and the expression of 55K protein in such cells was then studied. The results indicated that the amount of the protein decreased with increased differentiation and was not present in fully differentiated (parietal endoderm) EC cells. On the other hand established parietal yolk sac cell line such as PYS-2 were found to express the same protein. Thus it remains to be seen whether the detection of 55K protein could be correlated with expression or differentiation of any particular cell type.

B) 1. Expression of 55K Protein is not a General Correlate of Cellular Tumorigenicity. We present here a summary of experiments on three families (two AL/N and one Balb/c strain) of mouse clonal cell lines, and derivative tumor lines with respect to the expression of 55K protein.

The first family of cells originated from a spontaneously transformed highly malignant (median tumorigenic dose, $TD_{50}=10^2$ cell/mouse) AL/N mouse embryo cell clone 104C. We have shown previously that after SV40 transformation the tumorigenicity of 104C cells in immunologically competent syngeneic mice becomes lower ($TD_{50}=10^4$), apparently because of the expression of the SV40 T and transplantation antigens. One such "daughter" cell, the SV40 T antigen positive clone *106CSC was shown to contain 1 copy number equivalent of SV40 DNA per cell DNA. When we injected *106CSC cells into the syngeneic mouse, a negative immunologic selection resulted in T antigen negative revertant derivative tumor lines and clones that originated from a rare cell(s) through some DNA rearrangement. These T antigen negative revertants (i.e., the 124, 127, 128 CSCT

and 134SCTC cells) retained any "late" SV40 DNA sequences, and probably also a region (Taq 1 - Hha 1, 0.73 - 0.55 map units) which includes the sequence coding for small t antigen (0.65 - 0.55 map units). In the tumor lines and clones (128, 127, 124CSCT and 134SCTC) negative for large T antigen there was no specific 55K MW protein band detectable, just as in the distant parent 104C cell before the SV40 transformation, while the proximal parent T antigen positive *106CSC cell possessed the 55K protein. Thus the presence of one copy number equivalent of early SV40 DNA encompassing sequences coding for large T (but not necessarily for small t) antigen, and the resulting synthesis of SV40 large T antigen, is required and is sufficient in this family of mouse embryo fibroblast cells for the production of the 55K cellular protein. Our results with the T antigen revertant cells extend the findings of others by demonstrating clearly that the continued presence of the A gene and synthesis of large T antigen is required for the maintenance and production of the 55K cellular protein. In SV40 transformed cells large T antigen is shown to form a complex with 55K protein and therefore the precipitation of 55K could be due to this complex formation. However, it must be noted that the serum from SV40 tumor bearing hamsters, used thorughout these experiments, precipitated the 55K cellular protein alone from the F9 EC cells and from the embryo cells. After extracts of labelled SV40 transformed cells were immunoprecipitated and separated by gel electrophoresis, separate bands of the gel at the 55K and 94K region were cut out, eluted, re-precipitated by the tumor serum, and re-electrophoresed. The appropriate 55K or the 94K proteins immunoprecipitated. Thus the serum from SV40 tumor bearing hamster used by us contained specific antibodies against the 55K cellular protein. When excess amounts of unlabelled extracts of *106CS cells, which contain T antigen were mixed with the extracts of ^{35}S-methionine labelled 124CSCT cells, there was no more radioactivity precipitated in the 55K region with the SV40 hamster tumor serum than with the control normal serum. This could mean either that there was no complex formation in mixed extracts between the large T antigen of *106CSC cells and the 55K protein of labelled 124CSCT cells, or that the 55K protein is not present in the extract of labelled 124CSCT cells. Since the serum from SV40 tumor bearing hamster used is capable of precipitating large T antigen from SV40 transformed cells, and independently the 55K cellular protein from F9 and embryo cells in the same extraction buffer as in the mixing experiment, and in buffers with other detergents in the separation and re-precipitation experiments. These results are interpreted to mean that the highly malignant ($TD_{50} \leq 10^2$) 124CSCT and all the other T antigen negative cell lines do not synthesize (detectable amounts of) 55K cellular protein.

The second family of cells studied originated from a clone (210C) of AL/N mouse fibroblast cells which possessed very low tumorigenicity ($TD_{50}=10^{6.5}$). However, when injecting 10^7 210C cloned cells, tumor lines of high tumorigenicity ($TD_{50}<10^2$) can be obtained, such as the 219CT. The simplest explanation is that in 210C cells, variant (mutant) "spontaneously" transformed highly tumorigenic cells(s) arise at low frequency, and these cells are selected for tumorigenicity by the in vivo passage. The highly tumorigenic 219CT cells possess no 55K MW cellular protein. When the 210C cells were infected with SV40 and immediately recloned, the derivative T antigen negative daughter clone 213CSC had no 55K MW cellular protein, while the independently transformed SV40 T antigen positive daughter clones *214CSC and *215CSC both had the 55K cellular protein. Note that the transformation of 210C with SV40 resulted in the T antigen positive

*214CSC and *215CSC clones which have similar low tumorigenicity as the 210C. From 10^7 *215CSC cells the tumor lines obtained from separate mice all had high tumorigenicity ($TD_{50} 10^2$-10^3), irrespective of whether they were T antigen positive (*221CSCT, *222CSCT, and *224CSCT) or T antigen negative (223CSCT); the 223CSCT being probably a similar revertant to those discussed in the first cell family. Only the SV40 positive clones were shown to contain 55K protein.

In the third cell family, derived from a Balb/c strain tumor, the highest relative tumorigenicity was the property of the parent line 301 T, which was the only SV40 T antigen negative line and that was also the only line that did not contain the 55K protein. Thus all the spontaneously transformed malignant sarcoma cells tested from AL/N and Balb/c mice did not contain the 55K cellular protein. The tumorigenic and non tumorigenic cell lines synthesize small amounts of 55K (about 2%) when compared to SV40 transformed cells. We conclude that the presence (synthesis) of the 55K cellular protein is not a correlate of spontaneously arising tumorigenicity (malignant sarcomas) in established mouse fibroblast cells, but it is an absolute correlate of SV40 infection and transformation.

2. Half Life Determination. The absence of detection of this protein in tumorigenic cell lines could be due to differential turnover of this protein in these cells and the SV40 virus transformed cell lines. To study this the cells were labelled for a short period of time (30 min.) and then chased for various periods of time. The cell extracts were then immunoprecipitated and the 55K band was quantitated either by direct counting or by integrating the peak area. The results showed that during a 30 min. pulse label all the non-tumorigenic and tumorigenic non-SV40 transformed, as well as SV40 transformed cell lines showed the presence of 55K protein but the amount of much lower in the non-transformed cell lines compared to SV40 transformed cells. The half life of the protein was also found to be different. The 55K in non-SV40 transformed tumorigenic and non-tumorigenic cell lines had a half life of about 60 minutes, whereas the 55K in SV40 transformed cells had about 300 minutes.

C. Subcellular Distribution and Heat Stability of 55K Protein in SV40 Transformed Cells. The subcellular distribution of this protein was then studied in SV40 transformed cells to locate the 55K protein. Our initial studies on subcellular fractionation indicated the presence of 55K protein both on nucleus and on the surface of SV40 transformed cells. We utilized the following biochemical studies to confirm these observations.

a) Trypsin treatment of metabolically labelled intact SV40 transformed cells to determine the proteins released from the surface of these cells showed the presence of both large T and 55K on the surface.

b) Lactoperoxidase catalyzed iodination from the outside showed that both large Tag and 55K were iodinated by this method and these proteins were then digested preferentially by trypsin.

c) Large T antigen in cell extracts were found to be less heat stable compared to 55K protein and the 55K protein was precipitated in these cell extracts without the precipitation of large T antigen. Such heat treated cells were then shown to protect mice when immunized against SV40 induced tumor development.

Significance to Biomedical Research and the Program of the Institute:
Re-expression of embryonic antigens in transformed cells have been used in immunological protection studies against tumor development. Analysis of the identification of these proteins would provide more insight into the mechanism of transformation and the phase specific expression of this protein could be used at the molecular level to study embryonic differentiation.

Proposed Course of the Project: Attempts will be made to determine whether any particular cell type from embryonic cells could be isolated that would synthesize the protein constitutively or to find out whether this protein would serve as a marker of differentiation of embryonic cells. In this regard, in collaboration with Drs. Solter and Edidin, we plan to detect this protein in pre-implantation embryos. We also plan to use the monoclonal antibodies against 55K to detect the presence of this protein in spontaneous transformed tumorigenic cells. The nature of phosphorylated amino acid in the 55K protein from both SV40 transformed cells and embryo cells will be studied in an attempt to find its biochemical role in transformation and embryonic development.

Publications:

Mora, P. T., Chandrasekaran, K., and McFarland, V. W.: An embryo protein induced by SV40 virus transformation of mouse cells. Nature 288: 722-723, 1980.

Chandrasekaran, K., Winterbourne, D. J., Luborsky, S. W., and Mora, P. T.: Surface proteins of SV40 transformed cells. Int. J. Cancer 27: 397-407, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05526-13 IP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Cell Surface Changes in Spontaneously or SV40 Transformed Mouse Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	S. W. Luborsky	Chemist	IP	NCI
	V. W. McFarland	Chemist	IP	NCI
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COOPERATING UNITS (if any)
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the cell surface membrane, how it may change in transformation to malignancy, either by spontaneous induction, or as induced by SV40 virus; to study the nature of biochemical and biologic changes associated with the cells being transformed by either SV40 or by spontaneous event(s).

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To elucidate certain biochemical and biological changes both in the cell surface and in the control mechanisms involved in cell division, which occur during transformation to malignancy in mouse cells.

Methods Employed: Labelling glycoproteins, glycosaminoglycans and glycolipids, proteins and nucleic acids with radioactive precursors during cell growth in culture. Novel fractionation techniques for isolation of (labelled) subcellular components, including cell surface membranes and macromolecules associated with surface membranes. Isolation and characterization procedures include thin-layer and column chromatography, gel electrophoresis, electrofocusing and autoradiography of gel slabs. Analytical and preparative ultracentrifuge studies. Enzymological techniques pertinent to intermediary metabolism. Cell growth dynamics in culture, and also in vivo, in syngeneic and in nude mice.

Major Findings: I. Analysis of Glycosaminoglycan Metabolism. We have previously shown that, after infection of a "parent" cloned mouse cell (210C) by SV40, the metabolism of heparan sulfate is altered only in the subclones which possess SV40 DNA and express the SV40 T-antigens, but not in an untransformed T-antigen negative "sister" clone. Such changes in heparan sulfate were first reported by others in SV40-transformed 3T3 mass cell lines selected for their ability to form foci in cell culture. Thus, the altered metabolism was detected consistently in those cells which express the SV40 T-antigens, the proteins thought to be responsible in transformed cells for stimulation of DNA synthesis and for loss of growth control.

As an alternative to the virus transformation model, we have isolated inter alia two highly tumorigenic tumor cell lines: 219CT and 220CT by in vivo selection from two independent "normal" clones (210C and 216C respectively) of AL/N mouse fibroblasts which possess very low tumorigenicity. Because these tumor cell lines were isolated from tumors induced in the syngeneic mouse by injecting 10^7 or 10^6 cloned cells, and because the tumor lines possess heritably very high tumorigenicity (median tumorigenic dose $<10^2$ cells/mouse), the simplest explanation is that they represent variants (possibly mutants) which arose spontaneously by a chance event at a very low frequency, and which must have been selected out from the clones on the basis of their high tumorigenicity. We reasoned that, if there is a specific biochemical difference which shows up in between at least two spontaneously transformed tumor cell lines and their respective parent clones, which is also the same between the parent and the SV40-transformed daughter clones mentioned above, this is unlikely to be just a coincidence, as both models of transformation select very rare transformed cells by presumably quite different mechanisms, but most likely it reflects basic and common biochemical alterations in both types of transformation events.

First we confirmed that the same specific alteration in anion exchange properties of glycosamine and glycosaminoglycans as was previously reported for SV40 transformed subclones, was found in both 219CT and 220CT. One tumor line (219CT) and one SV40 transformed subclone (215CSC) were then compared with their common parent clone (210C). The preliminary finding was discouraging: cellulose acetate electrophoresis at pH 1.0 showed that 215CSC heparan sulfate had a slight overall decrease in sulfation compared with heparan sulfate from 210C, however no

gross difference could be detected between heparan sulfate from 219CT and 210C. However, analysis of the products of deaminative cleavage of heparan sulfate by nitrous acid under conditions where cleavage occurs quantitatively at N-sulfated glucosamine residues showed that although heparan sulfate from the three cell lines gave similar yields of O-sulfated disaccharides, both 215CSC and 219CT had only about half as many O-sulfate residues in higher molecular weight oligosaccharides compared to heparan sulfate from 210C. Then an enzymatic degradation of heparan sulfate with a mixture of enzymes from F. heparinum showed that heparan sulfate from 215CSC and 219CT had 30% fewer glucosamine residues bearing 6-O-sulfate groups. This is a specific reduction in 6-O-sulfate glucosamine residues which occurs in regions of the chain containing relatively few sulfate groups. It became clear that certain sequences of charged groups present in heparan sulfate from 210C will be found only rarely in heparan sulfate from 215CSC and 219CT, and that this very specific biochemical change is common to both the SV40 transformation and to the spontaneous transformation.

The biochemical findings have been correlated with the biological properties. It should be considered that the altered metabolism of heparan sulfate is related to the ability to grow to high cell densities, as SV40 transformants such as SV3T3 are selected for this ability, and the SV40-transformed cells studied here, although isolated without bias to their growth properties, also were found to have high saturation densities. However, the altered anion exchange property was also observed in the tumor cell line 220CT compared to its parent 216C even though the cell lines have been found to possess similar high saturation densities. Furthermore, the cell growth properties in culture did not correlate with tumorigenicity. This was particularly true for the plating efficiency in methocel (anchorage independent growth) which is generally considered to correlate more closely with tumorigenicity than other cell parameters, and yet this growth was very low for both spontaneously transformed highly tumorigenic tumor cell lines. In addition, we know from our earlier work that transformation by SV40 does not necessarily result in increased tumorigenicity in mouse cells, as the SV40-coded T and tumor-specific transplantation antigen on the cell surface causes recognition and rejection of cells in the syngeneic mouse. However, after SV40 infection of nonpermissive cells, there is in coincidence with the expression of T-antigens an invariable increase in the synthesis of cellular DNA accompanied by increased activities of the appropriate enzymes. Of course it is commonly thought that any tumorigenic transformation must be associated with loss of control of DNA synthesis. Therefore we postulate that it is in this respect, over-riding the normal control of metabolism, a putative element in cell growth control, is somehow connected with the two different forms of "transformations" studied by us.

The biochemical part on this project, the glycosaminoglycan synthesis and related enzymatic changes, the studies on the putative coupled enzymatic changes in DNA biosynthesis have been discontinued in this laboratory since Dr. Winterbourne left. However, we continue to collaborate with Dr. Winterbourne in London, in the selection and the biologic characterization of further (clonal) cells useful for his studies (see below).

II. An Approach to Analyze the "Spontaneous" Transformation of Cells. Obviously there are formidable theoretical odds against a systematic analysis. Yet, during the last five years we carefully developed "families" of closely

related mouse clonal cells, which we believe allows now a rational and unique approach.

The original cause of this work was sheer frustration: with cells obtained from (and selected by) others, many of the biochemical correlates we found (cf. the ganglioside changes with R. Brady) turned out to be more of the function of some tissue culture "selection" process, rather than a general correlate of the cellular tumorigenicity. Incidentally, this probably affects all of the results of biochemists and molecular bologists much more than it is generally recognized, except when conditional (lethal) mutants (cf. ts mutants) of viruses are employed as transforming agents. For a meaningful study of "spontaneous" transformation close and controlled genetic relatedness of cells, statistically significant number of subclones, very careful parallel handling of cells (cf. equal number of passages in culture), and realization and controlled use of the potential selective in vitro and in vivo factor(s) is all essential.

First we selected from an AL/N embryo mass cell line two "parent" clones of mouse fibroblast. One clone (104C) had very high tumorigencity ($TD_{50}<10^2$), the other clone (210C) very low ($TD_{50}=10^{6.4}$ cells/syngeneic mouse representing an essentially normal cell). The 104C cells and its clonal derivatives were and are still most useful in studying the innate spontaneous tumorigenicity, and in the case of SV40 transformed derivative subclones through the expression of the SV40 T and TSTA antigens on the cell surface, the effect of the SV40 coded antigens in functionally opposing the spontaneous tumorigenicity. For example, a series of publications during the last five years led to several important findings: The T antigen of SV40 is the TSTA; T antigen and early SV40 DNA minus revertant cells (clones) can be selected out by transplantation through immunocompetent syngeneic host (this obviously is preceeded by DNA rearrangement which leads to the loss of the early SV40 DNA) etc. The 104C parent clone, in fact had too high degree of DNA "instability" and too high frequency of "spontaneous" transformation to tumorigenicity, thus it was not realistic to use it as an average candidate cell for transformation analyses.

The parent clone 210C (and another clone 220C) were found ideal, however, in their (putative) mutational frequences to tumorigenicity ($1/10^6$ - 10^7). From the 210C clone alone we have isolated 10 independent tumor lines by a single in vivo passage (work with L. Waters): each tumor line has $TD_{50} \simeq 10^2$, both in the syngeneic and in nude mice, and this phenotypic property is heritable and stable. By recloning (with V. McFarland) one tumor line we obtained 14 subclones, and we are in the midst of the biologic characterizations. We have preliminary results which encourages us to believe that this family of cells represent an unique collection to study (on a statistically significant level) the biochemical correlates of spontaneous ("chance") transformation on various molecular levels (DNA, RNA, proteins etc.). To exploit these cells fully collaborative work is being set up with many laboratories in this country and abroad (for example transfection of the non-tumorigenic parent 210C with the restriction endonuclease DNA fragments of the derivative tumor clones, etc.).

We wish to bring up one interesting biologic result: the highly tumorigenic mouse clonal cells, as a rule, do not grow without anchorage in viscous medium. Thus while cells which grow well without anchorage are generally tumorigenic, the opposite is not true. Obviously, for in vivo tumor growth further "factors"

are required in the interaction of these cells with the host, which do not show up in the anchorage independent in vitro growth experiments in the viscous medium. Our cells therefore also appear uniquely useful to study such in in vivo factors, both in the syngeneic and in the nude mice.

Third type of cell families, originating from Balb/c strain mouse embryo fibroblast clones, are also under study. Several derivative subclones were obtained (as in the previously described AL/N cell families too) both after spontaneous and after SV40 induced transformations. Numerous clones which were obtained by independent SV40 transformations have been most valuable in the SV40 T antigen studies, and in the SV40 induced 55K protein studies (see below).

III. SV40 Transformation, Cell Biology and Surface Antigen (Protein) Studies. The analysis which led to an absolute correlation of the expression (detectability) of the cellular 55K protein with the presence of functional T antigen in the above referred numerous clonal derivative cell lines has been reported in original papers and in reviews; the recent results are summarized by Dr. Chandrasekaran (Z01 CB 05545-01 IP). The studies on the binding of the 55K protein and of T antigen to DNA, and the presence of these proteins on the cell surface is reported by Dr. Luborsky (Z01 CB 05544-13 IP). The identification of the embryo 55K protein with the SV40 induced 55K protein is in collaboration with D. Simmons, University of Delaware, and the major results are reported in Z01 CB 05545-01 IP, and in Z01 CB 05546-01 IP.

Dr. C. Chang, in Taiwan, has completed a collaborative study on the SV40 T and surface antigen recognition by T cells, he also employs the cells in further collaborative work on (other) tumor specific antigen studies in human biopsies, similar to Dr. Coll in Madrid (see Z01 CB 05545-01 IP).

We supply well characterized clonal cells to numerous investigators in this Country and abroad: Timothy Rose is finishing a doctroal thesis on the SV40 cell surface antigens using our SV40 transformed clones in the Dept. of Molecular Biology in Geneva under Professor R. Weil; Dr. David Lane, at Imperial College, London, studies the interaction of the SV40 T antigen and the 55K cellular protein; Dr. D. Winterbourne at the Dept. of Biochemistry, St. George's Hospital Medical School also at London, studies the glycosoaminoglycan and DNA biosynthetic enzyme changes (see above); Dr. W. Deppert at the University of Ulm, West Germany, studies subcellular distribution of the antigens, etc.

In this laboratory (Drs. Chandrasekaran and Luborsky) used the SV40 transformed clones to detect the SV40 T antigen and also the 55K protein in the cell surface (plasma membrane) enriched subcellular fraction. The accessibility of these proteins on the cell surface was confirmed by radioactive iodination as catalyzed by alctoperoxidase, from the outside of the cell, and also by susceptibility to removal by gentle trypsinization (Dr. Chandrasekaran). Currently Mrs. McFarland is developing fluorescent staining techniques for the detection of SV40 T antigen and for the 55K protein on the cell surface (in part in collaboration with Dr. Edidin, Johns Hopkins, and D. Solter, Wistar Institute).

Using the families of clonal cells, transformed spontaneously and/or by SV40, we find that spontaneous transformation (cf. by somatic mutation) is sufficient to explain the acquisition of cellular tumorigenicity of SV40 transformed mouse

fibroblast. When considering on the balance the in vivo phenotypic changes in mice, the SV40 early gene coded proteins are more "dominant" as cell surface antigens facilitating immunologic recognition and rejection, rather than causing tumorigenic transformation. There is no binding general correlation between cell growth in viscous medium, of tumorigenicity in vivo, and of the SV40 antigen expression, in the mouse fibroblast cells we have investigated. This of course does not mean that the SV40 early gene, its expression, and the role of the T antigen is not a suitable system to study the correlation between certain biochemical controls in cells and their phenotypic tissue culture growth, when such correlations can be made (such as between DNA synthesis and focus formation in culture). Our research, however, emphasizes a growing recognition that for the acquisition of tumorigenic potential the transformation by SV40 is not a sufficient cause, and not even a crucial contributory event. This is apparently true not only in mouse or rat cells, but also in hamster cells (cf. Lewis and Cook, PNAS, 1980).

Significance to Biomedical Research and the Program of the Institute: Studies of biochemical changes in cells, particularly in the cell surface, which relate to acquisition of tumorigenic potential caused by some rare event, such as somatic mutation, and the studies on immunologic recognition and rejection in vivo are of interest, especially when they may lead to understanding of the changes in molecular mechanism which appears to be reducible to heritable changes of control processes on the DNA level.

Proposed Course of the Project: Attempts will be continued to further clarify the relevance of the various changes in cell membrane biochemistry, especially in the heparan and chondroitin sulfates, to spontaneous and to viral induced transformation of cells. We also plan to relate these changes to phenotypic changes in cell immunogenicity and in tumorigenicity in various syngeneic and nude mouse systems. Studies on the molecular level will also include regulatory events which may be due to association of the SV40 A gene product with selective (e.g. nuclear membrane associated) portions of the cellular DNA. We will further study 1) the relationship between SV40 TSTA and the cell surface, 2) control of gene expression for the newly discovered 56K protein and its relation to transformation and its role in embryogenesis, 3) the basis for the interaction of the 56K cellular protein with the various antisera, including monoclonal hybridoma sera, 4) we will also attempt isolation of the transforming allele(s). We hope that the information and emthodology developed in the mouse system will be of use in studies on human tumor cells.

Publications:

Simmons, D. T., Martin, M. A., Mora, P. T., and Chang, C.: Relationship among TAU antigens isolated from various lines of simian virus 40-transformed cells. J. Virol. 34: 650-657, 1980.

Chandrasekaran, K., Winterbourne, D. J., Luborsky, S. W., and Mora, P. T.: Surface proteins of simian virus 40 transformed cells. Int. J. Cancer 27: 397-407, 1981.

Winterbourne, D. J., and Mora, P. T.: Cells selected for high tumorigenicity or transformed by simian virus 40 synthesize heparan sulfate with reduced degree of sulfation. Biol. Chem. 256: 4310-4320, 1981.

Mora, P. T., and Chandrasekaran, K.: Role of SV40 Induced Antigens in Transformation and Rejection of Malignant Mouse Cells, and the Detection of an Embryo Protein. In Manson, L. and Nowotny, A. (Ed.): Biomembranes. New York, Plenum Publishing Corporation, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05546-01 IP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
A Common Protein in Embryonic Differentiation and in Cellular Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. T. Mora	Chief, Macromolecular Biology Section	IP	NCI
Other:	K. Chandrasekaran	Visiting Fellow	IP	NCI
	V. W. McFarland	Chemist	IP	NCI
	C. Parrott	Chemist	IP	NCI
	T. M. Martensen	Staff Fellow	LB	NHLBI

COOPERATING UNITS (if any)
D. Simmons, University of Delaware; E. G. Gurney, University of Utah; M. Dziadek, Kansas State University; J. Coll, Instituto Nacional de Prevision, Madrid, Spain

LAB/BRANCH
Immunology Program

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	.5	1.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A specific 55,000 MW phosphoprotein was found in murine embryo cells. This protein is very similar, if not identical, to an SV40 induced protein, isolated earlier from SV40 transformed cultured fibroblasts. The amount of the embryo phosphoprotein is highest in cells taken from midgestation stage mouse embryos. We are studying the biochemical nature and the possible function(s) of this embryonal protein. This (or similar) protein(s) appear in cultured cells which were transformed by various other means including RNA viruses, and also in much lower amounts in normal or spontaneously transformed cells. The presence of protein in high amounts is not a general correlate of cellular tumorigenicity.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To isolate and characterize a specific cellular phosphoprotein of ~55,000 MW which is present in midgestation stage embryo primary cells and also in many cells transformed by various agents, and to find its biochemical function.

Methods Employed: The biochemical and tissue culture methods are outlined in Project Report Z01 CB 05526-13 IP. In addition, complex immunochemical techniques, such as detection of cell (surface) antigens both in cell culture and in situ in embryos with pertinent specific and monoclonal antibodies, dissection of mouse embryos of various ages for organ precursors.

Major Findings: I. Related results are presented in Z01 CB 05545-01 IP by K. Chandrasekaran (it is necessary to summarize these here, to understand the further findings).

1) The isolation and quantitation of the 55K embryo protein from 10-13 day old mouse embryos.

2) Identification of this protein with a monoclonal antibody induced against the 55K protein in SV40 transformed mouse fibroblast cells.

3) The decrease of the embryo protein in mouse embryo primaries, after the embryo was 14 days old or older.

4) Detection and characterization of a similar 55K protein from midgestation age hamster embryos, and demonstration that it is the same protein which is induced in SV40 transformed hamster cells.

5) A loss or decrease of the embryo protein after replating primary cultures.

6) Absence, or presence only in very low amounts of (stable) proteins in normal mouse cloned cells or in spontaneously transformed mouse cells which readily induce fibrosarcoma.

7) A short half-life (fast turnover) of this protein in normal or spontaneously transformed cells.

8) Absolute correlation of the detectability of the protein with the expression of the SV40 T antigen in SV40 transformed clonal cells and in their revertants.

9) Presence of a small amount of this protein on the cell surface of SV40 transformed cells. This is discussed in detail in Z01 CB 05544-13 IP by S. W. Luborsky.

10) Correlation of the 55K protein with the differentiated state of the mouse embryonal carcinoma cells such as the F9 cell line.

II. Further major findings on the 55K cellular protein:

1) The embryo protein is a phosphoprotein. From 14 day old rat embryo primary cultures, labelled with ^{32}P, the 55K protein was specifically precipitated with a monoclonal antibody prepared against the 55K protein from SV40 transformed mouse fibroblast (monoclonal Ab from E. Tucker Gurney, University of Utah). This result was consistent with two of our earlier findings (with D. Simmons, University of Delaware): a) the mouse and rat cells transformed by SV40 produce 55K proteins with many (but not all) ^{35}S-methionine labelled tryptic peptides apparently identical and b) the SV40 induced proteins in a given species and the embryo proteins in the same species give very similar (near identical) peptide fingerprints.

As almost complete identity in the fingerprints were observed between the 55K proteins from SV40 transformed mouse cells and mouse embryo primary cells (which were not exposed to SV40), the easily obtainable 55K phosphoprotein from SV40 transformed mouse cells was chosen for further study. The pure 55K phosphoprotein from the latter cells, labelled by $^{32}PO_4$ appears to possess phosphoserine residues and some phosphothreanine residues, but not detectable amounts of phosphotyrosine residues (with T. Martensen, LB, NHLBI). Recently published results by others (G. Jay et al.) in labelling with $\gamma^{32}P$ ATP (indicating associated phosphokinase activity) in other SV40 transformed mouse cells is consistent with these findings. Concurrently and independently, we also showed an cAMP independent $\gamma^{32}P$ ATP phosphokinase activity associated with the 55K protein (with C. Parrott). We are now involved in determining what type of phosphorylation and phosphokinase activity is present in embryonal carcinoma cells such as in F9 cells, and in embryo primary cells. Since the phosphorylated amino acid is mainly serine, the 55K protein isolated by us is apparently different from the 55K MW phosphokinase intermediate (pK_S) of the protein kinase cascade in Ehrlich ascites membranes which regulates the phosphorylation of Na+ K+-ATPase through the tyrosine residues (recently elucidated biochemically by Racker, and found by other investigators to have pertinence to numerous RNA virus induced transformations of cells). We are exchanging information and reagents with the pioneer groups (E. Racker and D. Baltimore), and are in the midst of experiments (with T. Martensen) in elucidating the biochemical and biologic correlates of the phosphokinase activity of the 55K embryo protein(s).

2) Distribution of the 55K embryo protein during embryogenesis. Attempts are being made to develop detection techniques in various organs and in various phases of the developing embryos. The 13 day or 15 day old mouse embryos were freed of their extraembryonic membranes, and then dissected to provide various organs for labelling with ^{35}S-methionine (with M. Dziadek). The organ cultures of 13 day old mouse brain, liver and carcass showed the presence of the 55K protein, but not of the 15 day old mouse embryos. This is consistent with the drastic decrease of the 55K protein found in embryo primary cultures from 16 day old mice, as compared to the 12 day old embryo primaries (I. 3).

Immunofluorescent methods are being developed to stain in situ sections of (mouse) embryos in different stages of development, using fluoroscein labelled monoclonal antibodies (with V. W. McFarland in collaboration with Davor Solter, Wistar Institute).

3) Cell division rate and the 55K protein. The amount of the 55K protein in embryo primaries was the same when the ^{35}S-methionine labelling was in the presence of 2% or 5% FCS, and the decrease of the 55K protein by embryo age was also unaffected by such variations in serum concentration. Increase in FCS concentration is known to increase cell growth rate, at least in established cell lines. In established clonal normal mouse lines increase in serum concentration (from 2% to 10% FCS) increased somewhat ($\leq$10%) of the very low amount of the detectable 55K protein present ($\leq$2%, compared to its SV40 transformed derivative clone). Careful cell growth rate measurements and quantitations of the 55K protein are in progress in numerous established normal and transformed cell lines and clones, and also on embryonal carcinoma cells. Up to this time no general correlation is being observed with cell growth rate. It is important to emphasize that our experiments on embryo primaries (as in any other cells) are all in rapidly dividing cells close to their optimal growth rate. Under such conditions the effects of other parameters (such as the age of the embryo, difference between primary and secondary cultures, the presence of SV40 T antigen, transformation (and selection) of certain cells are all much greater (50-100 x higher), than the small effects (<10%) of cell division rate on the amount of the 55K protein in the cells.

4) The detection and possible biologic correlates of the 55K embryo protein in human tumors, and in various (transformed) mouse cell lines.

A) As many (but not all) established human tumor lines were shown by others to contain the 55K protein in well detectable amounts, we embarked on a collaborative screening project on the detectability and quantitation of this protein in freshly established (primary) cells from various human tumor biopsies at a large research hospital in Madrid (Instituto Nacional de Prevision) which has special capacity and competence of this work. The Department of Pathology and Immunology of the hospital has access to large numbers of biopsies of various malignancies, and occasionally of small amounts of (adjacent) apparently normal tissues. The Head of the Cancer Cell Membrane and Immunology Research Section in the Department (Dr. J. Coll) was well trained in the required techniques, beginning at the Rockefeller Institute than at Johns Hopkins and finally in our laboratory: Dr. Coll was the first researcher in this laboratory who established techniques and published careful PAGE analyses of "Metabolically labelled cell membrane proteins in spontaneously and in SV40 virus transformed mouse fibroblasts" (Coll, Luborsky and Mora, Biochemistry 16: 3169, 1977). Now we are supplying Dr. Coll with the specific antibodies necessary for the detection of the 55K protein, and with information on optimizing quantitation of the 55K protein. As yet this collaborative project is in too early a stage to say whether there is any utility to follow the quantitation of this new (class of) embryo "antigen(s)" for diagnosis of any human malignancies.

B) More careful analysis of the question whether the presence of (stable) 55K protein in large amounts is a correlate or not of cellular tumorigenicity is being carried out in our laboratory using newly established clonal mouse cell lines with careful pedigree and close familiar relationships. In mouse fibroblasts from three "families" of cells no correlation was detectable with the TD_{50} values in syngeneic (AL/N or Balb/c) or in nude mice (cf. B) 1 of Z01 CB 05545-01 IP). Several highly tumorigenic clones ($TD_{50} \leq 10^2$) form rapidly ($\leq$8 weeks) lethal fibrosarcomas similarly to the normal parent clone ($TD_{50} > 10^6$)

possessed very little 55K protein (1/50th to 1/100th of that in SV40 transformed derivative clonal cells). Thus in these mouse fibroblasts, there is no correlation with tumorigenicity in either the syngeneic or in the nude mice.

However, we found other mouse cells (clones), which are considered malignant, which posses the 55K protein in ample amounts. These cells (in addition to RNA virus or methylchorantrane transformed cells investigated by others) include a mouse neuroblastoma clone (neuro 2 a) and an L cell clone (#929). All of these cells, however, do not have closely matched normal counterparts, and have been in culture for a long time.

Clearly our newly isolated and characterized families of cloned cells with careful pedigree are the choice for further controlled biochemical and biologic studies.

Significance to Biomedical Research and the Program of the Institute: Re-expression of certain embryonic antigens in some cancers have been used by others in immunological studies concerning development of certain tumors. Analysis and identification of the embryo protein(s) we have discovered could provide more insight into the mechanism of transformation; also the phase specific expression of this protein could be used at the molecular level to study embryonic differentiation.

Proposed Course of the Project: Specific plans and work underway are given in Z01 CB 05545-01 IP. On a general level those potential avenues will be explored which apply the molecular biology techniques and reagents so well defined in SV40 virology to changes which occur in embryogenesis, and may also relate to control of cell growth rate of both normal and malignant cells. It is hoped to define the cellular gene which is constitutively expressed in normal embryogenesis, the modulation of which occurs in many transformation processes. This is a new type of such cellular gene, different from those (such as the *src* gene) which have been found to be the cellular homologues of certain RNA tumor viruses: the cellular gene for the 55K protein is not "picked up" in the DNA virus genomes; its expression (at a yet unknown level or levels) is altered (controlled) in many different types of transformations, including the Abelson murine leukemia virus transformed B cells, the methylcholantrane induced mouse tumors, but not the spontaneous transformation of mouse fibroblast. Various potential molecular mechanisms for the modulation of the gene expression will be explored. The nature of the phosphokinase activity in embryo cells and in embryonal carcinomas will be studied by biochemical and immunologic techniques.

Publications:

Mora, P. T., Chandrasekaran, A., and McFarland, V. W.: An embryo protein induced by SV40 virus transformation of mouse cells. Nature 288: 722-724, 1980.

Mora, P. T., and Chandrasekaran, K.: Role of SV40 Induced Antigens in Transformation and Rejection of Malignant Mouse Cells, and the Detection of an Embryo Protein. In Manson, L. and Nowotny, A. (Ed.): Biomembranes. New York, Plenum Publishing Corporation, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05544-13 IP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cell Surface Changes in Transformed Mouse Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. W. Luborsky	Chemist	IP	NCI
Other:	K. Chandrasekaran	Visiting Fellow	IP	NCI
	P. T. Mora	Chief, Macromolecular Biology Section	IP	NCI

COOPERATING UNITS (if any)

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Immunology Program

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.0	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A study of changes on a molecular level which control cell properties, growth and differentiation, and are possibly involved in the transformation to malignancy as well. A cellular protein found bound to SV40 T antigen (TA) in cultures of SV40 transformed murine cells, and later in normal midgestation murine embryos, was thought, by analogy to TA, also to be involved in control reactions pertaining to DNA replication and cell growth and division. Since TA is known to bind to DNA, the behavior on calf thymus DNA-cellulose columns of extracts of cultured mouse cells was analyzed for information concerning the nature and properties of this 55,000 molecular weight (MW) protein (55K protein) in the presence of TA. Results were compared with those obtained from similarly treated extracts of embryonal carcinoma (EC) cells, which contain the 55K protein. Evaluation of the ability of the 55K protein (complex) to associate with DNA should provide a basis for understanding such interactions within the cell. It is hoped to gain a better understanding of the biological activity and function of such proteins and their contribution to the basic control mechanisms of the cell, as well as to develop better methods for their isolation and purification.

Project Description

Objectives: To gain some insight into the biological activity and function of proteins involved in the basic control mechanisms of cell growth and division and the maintenance of the normal phenotype, and to study the interaction and regulation of cellular and viral control proteins; to improve methods for their isolation and study.

Methods Employed: Tissue culture cell cultivation; cell cloning procedures; polyacrylamide gel electrophoresis; column chromatography, including DNA-cellulose chromatography; ultracentrifugation; 2-dimensional thin-layer electrophoresis and chromatography; various immunological assays to detect tumor antigens.

Major Findings: It is known that the tumor antigen (TA) of SV40 binds to SV40 DNA near the origin of replication and plays a role in the control of viral DNA replication and gene expression. It also has been shown to bind to cell DNA, although its binding site(s) have not been shown to be specific on this DNA. It nevertheless is thought to be responsible for malignant conversion and the maintenance of the transformed state by its effect on cell control mechanisms for growth, division and differentiation. Since TA was known to be involved in control mechanisms concerned with viral replication, and presumably with modifying and/or controlling the infected cell process, the discovery of a host protein, somehow derepressed/stimulated by the viral infection, which was specifically associated with the viral TA provided a basis for the expectation that this host 55K protein too was somehow involved in similar control processes in the normal cell. Such a presumption was supported by the detection of this 55K protein in normal, uninfected, midgestation murine embryos. It was felt that the 55K protein represented a contact point in the regulatory pathways of both the normal cell and the virus-cell systems.

One may study the 55K protein by attempting to clone its gene first, as some workers are currently attempting. More direct, and perhaps more easily interpreted, however, is the study we describe here of this 55K protein obtained directly from the cell, and its effects on the cell.

Our recent work has focussed principally on this 55K protein. We had shown that extracts of ^{35}S-methionine labelled SV40 transformed cells of mouse, hamster and rat contained anti-T serum reactive antigens of MW 94,000 and 19,000 (94K and 19K), the SV40 coded large T and small t antigens, respectively, and the host coded 55K protein, and that this 55K protein was to be found both on the surface and in the nucleus of SV40 transformed cells, in association with the 94K protein. Following reports of the presence in mouse embryonal carcinoma (EC) cell lines of a similar 55K MW protein, precipitated by the same hamster anti-T serum used with the SV40 transformed cells, we found this same protein also in midgestation stage murine embryos, not infected or transformed by SV40 (Z01 CB 05545; Z01 CB 05526). The nature and properties of this protein became a focus of major interest to us. We wished first to isolate the 55K protein in relatively pure native form for further study; to compare the 55K protein from the two cell systems for binding capacity to DNA; and to determine if binding was direct, protein to DNA, or if it required mediation of the 94K MW SV40 TA binding also.

For this purpose we chose the well characterized clonal culture line, 215CSC, an SV40 transformed mouse embryo cell, as an easily available source which we found contains a high concentration of the 55K protein. Since this protein had been found to be complexed with the SV40 large TA, known to bind to DNA, we attempted fractionation of an ^{35}S-methionine labelled cell extract on a DNA-cellulose column, using first calf thymus DNA. For a preliminary test, the 94K and 55K bands were eluted from a polyacrylamide gel electrophoresis (PAGE) pattern of the immunoprecipitated 215CSC mouse cell extract, passed through a DNA-cellulose column, and the fractions analyzed. While interpretation of the results was difficult because of the low levels of radioactivity available, a preliminary assessment indicated that the 94K SV40 TA was bound to the DNA and required a higher pH for its elution, while the free 55K cellular protein was not, and passed through the DNA column without binding, the latter being a negative result. It was felt that the 55K protein obtained directly from the cell extract may behave differently since it would not have been subjected to the various denaturing conditions involved in preparation and elution of the gel bands.

Subconfluent cultures of 215CSC cells in exponential growth were labelled with ^{35}S-methionine and the extracted proteins run on a DNA-cellulose column. Two to four times as much label as was bound did not bind to the column. When this unbound fraction was tested on a fresh DNA-cellulose column the ratio of unbound to bound material in it remained high (10-50 fold). Clearly the first column was not overloaded; the non-bound fraction did not contain any significant proportion of material that was capable of binding to DNA but had not during the first pass through the column. Nearly 10X more of the bound material than of the non-bound material was specifically immunoprecipitated by hamster anti-T serum, thus indicating the close correlation between DNA binding capability and anti-T serum reactivity. PAGE analysis of these immunoprecipitates revealed the usual 94K, 55K and 19K proteins present in both fractions, with the 94K and 56K species present in apparently somewhat similar amounts. Relative to the original proportions of radioactivity in both fractions, however, the bound fraction possessed over 6X more of both species than did the non-bound fraction. Moreover the 55K protein was present in both bound and non-bound fractions, a result contrary to the preliminary indication obtained from the 94K and 56K bands eluted from the gels.

The eluates were further analyzed by sucrose density gradient centrifugation, to determine the size distribution of the protein complexes as originally obtained from the cells, under less denaturing conditions. Generally, sedimenting peaks were seen at molecular weight positions of about $(17\text{-}22)\times10^3$, 45×10^3 and $(86\text{-}107)\times10^3$ daltons. Thus, in these solutions the size of various proteins present seems to correlate as uncomplexed proteins with the three major anti-T serum reactive antigens mentioned above. Work is continuing now to explore further the nature, amounts and properties of the 55K protein obtained from the DNA-cellulose column and the density gradient sedimentation procedures.

Another aspect of this study of the nature of the 55K protein derives from its presence in embryo cells not infected with SV40. An embryonal carcinoma cell, F9, was studied in much the same fashion as indicated above. Extracts of ^{35}S-methionine labelled EC cells were treated in this fashion and fractions which eluted from the DNA-cellulose column were analyzed. A large non-bound fraction

eluted first, following by 2 well separated bound peaks, the first eluted by the higher pH buffer (pH 8), the second, by this buffer containing 0.7M salt. The non-bound peak,and the loosely bound peak eluted at higher pH,both appear similar to those obtained from the 215CSC cell extracts, while the more tightly bound high salt peak was not seen before. Although the three peaks eluted from the DNA constituted 89%, 6% and 3%, respectively of the effluent, after dialysis, concentration and redissolving in buffer suitable for immunoprecipitation, 30%, 6% and 1.3%, respectively, remained. Following immunoprecipitation with hamster anti-T serum, samples are being analyzed by PAGE and fluorography. Interpretation of the nature and composition of these peaks awaits the results of this analysis.

Significance to Biomedical Research and the Program of the Institute: It is important to try to understand better the nature and properties of the cell components which exert important influences upon cell growth characteristics and the ability of certain cells either to replicate normally, to form tumors or be rejected by the host, or to differentiate in a normal orderly fashion. The 55K protein apparently provides a unique activity for study, representing as it does a contact point in the regulatory pathways of two systems, the normal cell and the virus-cell systems. As already pointed out, we have found this protein together with the SV40 TA, on the surface of SV40 transformed murine cells, as well as in their nuclei. The presence of such a regulatory protein on the surface may provide a link to mechanisms of surface control of cell growth and division. Many cell systems have recently been shown to possess this 55K protein, including cells of both virus infected or transformed or tumor origin, and uninfected normal cells, including midgestation murine embryos which we have studied (Z01 CB 05545; Z01 CB 05526). Such widespread occurrence underscores the apparent biological importance of this 55K protein (complex). It is hoped that evaluation of its ability to associate with DNA will provide a model for understanding better such interactions within the cell. These interactions may be the basis of the effect of some proteins in regulating differential cell growth and differentiation. Study of its behavior in these systems should contribute to a better understanding of basic cell processes at the molecular level.

Proposed Course of the Project: The nature of the binding of the 55K protein to DNA will be investigated, first to determine whether it can bind directly to DNA or requires a mediator, in the form of another protein such as the SV40 TA. Attempts will be made to fractionate the SV40 anti-T reactive proteins from various sources to obtain each free of the others and not denatured by high concentration of detergent, urea or other denaturing solvent, to compare the 55K proteins from each source. Particular attention will be focussed on possible differences in properties and/or function of the 55K protein obtained from virus transformed cells or from normal embryos or F9 EC cells. We will attempt to better understand the relevance of the various changes in cell biochemistry and immunochemistry, and the presence and function of the 55K protein, to spontaneous and to viral induced transformation, as well as to normal differentiation of cells.

Publications:

Chandrasekaran, K., Winterbourne, D. J., Luborsky, S. W., and Mora, P. T.: Surface proteins of simian virus 40 transformed cells. Int. J. Cancer 27: 397-407, 1981.

Annual Report of the Laboratory of Immunodiagnosis,
National Cancer Institute

October 1, 1980 - September 30, 1981

This report summarizes the activities of the Laboratory of Immunodiagnosis for its fifth full year, since its initiation in December, 1974. The research activities of the Laboratory of Immunodiagnosis can be divided into four main areas: Role of natural and induced effector and suppressor cells in resistance against cancer; Cell-mediated immunity against tumor associated and other antigens; Immunochemical studies of tumor associated antigens; and Application of immunologic procedures to clinical problems of cancer patients. The main objective of all these studies is the application of the research information to the diagnosis of cancer and the monitoring of disease in tumor bearing individuals. Each of the areas of research in this laboratory has been highly productive during the past year: There have been extensive studies on natural killer (NK) cells, with emphasis on the characteristics of these cells and their in vivo role in resistance against tumor growth. Several major new findings have resulted from the ability to isolate highly purified populations of human and rat large granular lymphocytes (LGL), a small subpopulation of cells which we have shown to be responsible for NK and antibody-dependent cell-mediated cytotoxic activity. LGL have been extensively characterized and shown to share features with T cells and also monocytes. Cytotoxic studies with the LGL have led to new insights into the mechanisms of their reactivity and of augmentation of their function by interferon (IFN). In addition to their potent cytotoxic activity, LGL have been found to also produce IFN in response to a wide variety of stimuli. The potential for charaterization and practical utilization of LGL has been expanded greatly by development of procedures to propagate these cells in culture and even to clone them. This has already provided an increased ability to discriminate between NK cells and immune cytotoxic T cells with anti-tumor activity. Further evidence has also accumulated in support of in vivo roles of NK cells, including their participation in immune surveillance against the development of chemically or radiation-induced tumors. Appreciable progress has continued regarding the cytotoxic, accessory and suppressor functions of macrophages and monocytes, and the processes involved in their activation. There has been a major increase in our emphasis on the production of somatic cell hybrids, to develop B cell hybrids marking monoclonal antibodies to subpopulations of human and mouse lymphoid cells and to human lung tumor associated antigens, and T hybrids secreting lymphokines. Several developments with considerable potential clinical importance have occurred: Considerable progress has been made in the purification of a human lung tumor associated antigen and in the development of a practical radioimmunoassay which can detect elevated levels of this antigen in the serum and urine of lung cancer patients. We have also developed the ability to monitor in detail and in a standardized manner the immunologic reactivity of cancer patients receiving various biologic response modifiers. This is currently being utilized to study the immunologic effects induced by purified recombinant leukocyte IFN.

Some Highlights of Achievements in the Last Year:

I. Role of NK Cells in Resistance Against Cancer

We have continued to perform extensive studies on natural cell-mediated immunity against tumor cells. During the past year, our studies have focused primarily on the following areas: a) the characteristics of NK cells and the

possible mechanisms for their cytotoxic activity; b) factors and agents affecting the levels of cytotoxic activity and the mechanisms for these effects; c) production of IFN by NK cells; and d) the relationship of NK activity to *in vivo* resistance against tumor growth.

A major advance in the characterization of human NK cells has come from the finding of their close association with LGL, that possess a distinct morphology with Giemsa staining, containing azurophilic granules, with pale, blue-staining cytoplasm. A considerable portion of these LGL form lytic conjugates with NK-susceptible targets. These cells have been isolated from peripheral blood lymphocytes using discontinuous Percoll density gradients and subsequent further purification by rosette formation with sheep erythrocytes at 29°C, to minimize contamination with small typical T lymphocytes. Using these procedures, we have been able to reproducibly obtain fractions containing >90% LGL by morphological analysis and containing <1% mature T cells as determined by morphology and monoclonal antibody analysis. Virtually all of the LGL have Fc receptors for IgG and they possess most, if not all, of the NK cell activity against NK-susceptible targets. We have extended our previous results by examining in detail a large variety of NK-susceptible targets, including both lymphoid and anchorage-dependent lines. A high degree of correlation existed between the presence of LGL and NK cytolysis of this large panel of targets. Also, fractions which were devoid of LGL could not be induced to become cytotoxic by the addition of activating agents such as IFN or IFN-inducers. Coupling our purification procedure with a single cell cytotoxicity assay, we have estimated that >70% of the LGL are functionally active NK cells. We have examined the blood or spleen cells of a variety of animal species for the presence of LGL and for the parallel expression of NK activity or antibody-dependent cell-mediated cytotoxicity (ADCC) activity. LGL and NK and/or ADCC activities have been detected in most of the species examined, including rats, mice, nonhuman primates, dogs, cats, sheep, cattle, pigs and chickens. In all of the species studied, the frequency of LGL among peripheral blood mononuclear cells varied between 1 and 10%. In parallel with the high NK activity in the blood or spleen of nude, athymic rats, LGL frequency was shown to be increased 2-5 fold in these rats.

Because of the ability to obtain highly purified human and rat LGL and their strong association with human NK cell activity, we were able to perform detailed phenotypic studies with these effector cells and compare them to the phenotype of typical small, mature T lymphocytes. This has been performed using a series of monoclonal antibodies, using the fluorescence activated cell sorter. A considerable proportion of human LGL reacted with antibodies OKM1 (60-80%) and anti-Ia (15-30%). 25% reacted with OKT8, which is similar to its expression on T cells. In contrast, however, very few LGL reacted with OKT3 or OKT4 antibodies, which react with a high proportion of mature T lymphocytes. OKT11A and 9.6 antibodies, which react with the sheep erythrocyte receptor, also reacted with approximately 50% of the LGL. However, OKT10 (which has been found to react mainly on pro- or early thymocytes) reacted with a high proportion of LGL, whereas none of the other peripheral blood lymphoid cell populations were positive. By depletion of the cells on antibody monolayers against the mouse monoclonal antibodies, we were able to determine that most, if not all, of the active NK cells were OKT10 positive and the majority were also OKM1 positive. A significant proportion (approximately 1/2) of the LGL were E-rosette positive as measured by 9.6 or OKT11A. An insignificant proportion of the functional NK cells possessed the OKT8 or Ia antigens.

As in the human studies, the rat LGL were found to be an antigenically distinct population of cells which share some characteristics with monocytes, T cells, and granulocytes. Essentially all of the LGL expressed the W3/13, OX-8, leukocyte-common (L-C), and asialo GM1 antigens. A portion of these cells were also positive for ART-1^a. In contrast, few LGL expressed Ia, W3/25, surface immunoglobulin, or Thy 1.1 antigens. Monocytes had a similar pattern but were OX-8 negative, and T cells expressed ART-1^a, W3/25 and/or OX-8.

A major limitation for the detailed analysis and characterization of NK cells is that they represent a small proportion of the lymphoid cells in the peripheral organs. A potential solution to this problem was suggested by the observation that peripheral blood lymphocytes can be cultured in the presence of partially purified T cell growth factor (interleukin 2 or IL-2) and these continuously growing cell lines have two distinct cytotoxic capabilities: 1) an NK-like and 2) a polyclonally activated T cell-like activity. These cultures have been shown to be cytotoxic against a variety of NK-sensitive targets and against alloblasts. Highly purified and enriched populations of LGL, which were essentially devoid of mature typical T lymphocytes, as well as T cell cultures, maintained rapid growth in the presence of IL-2. The T cells maintained relative stability in culture, not expressing new antigens as determined by a battery of monoclonal reagents, with the exception of the expression of Ia, which has been previously reported on activated T lymphocytes. In contrast, the LGL cultures demonstrated some very significant changes in phenotype. OKT10, OKM1, and Fc receptors were much dimished or absent by 10 days. Conversely, the appearance of Ia antigens and OKT3 antigens as well as the ability of a subset population of LGL cultures to form rosettes with sheep erythrocytes at 29°C became quite apparent at about the same time in culture. The cultured LGL demonstrated typical killing against NK-susceptible targets, and both fresh and cultured NK cells exhibited typical lectin-induced cytotoxicity. In contrast, the fresh and cultured T cells only demonstrated lectin-induced cytotoxicity and did not demonstrate NK-like killing against the panel of NK targets. Polyclonal activation against alloblasts as well as activation and killing against anchorage dependent target cells appeared in the cultured T fractions.

It has been possible to also maintain the proliferation of mouse cytotoxic cultured lymphoid cells (CLC) for 1-12 months in the presence of T cell growth factor. Clones from these cultured lymphoid cells were established by either limiting dilution or soft agar techniques. Most of the clones had strong cytotoxic activity against a variety of syngeneic and allogeneic tumor target cells. The clonal populations generally exhibited a more restricted pattern of cytotoxic activity than the parental CLC and the pattern varied among the clones. One group of clones reacted preferentially against lymphoid tumor target cells and the others reacted strongly against both lymphoid and solid tumor targets. The clones expressed some markers associated with NK cells (asialo GM1, NK2, T200) and also some characteristic T cell markers (Thy 1.2, Lyt 2).

Efforts have been made to determine the mechanism of cytotoxicity by NK cells. Substantial evidence was obtained for a role of serine proteases. Highly purified populations of human LGL, with no detectable contamination by monocytes, could be shown to produce a fibrinolytic enzyme with characteristics of plasminogen activator. Furthermore, addition of a wide range of selective protease inhibitors to the cytotoxicity assay resulted in strong inhibition of NK activity by some inhibitors. The pattern of inhibition was highly suggestive of an important role for a protease(s) with the characteristics of chymotrypsin. We have

also established a role for phospholipid metabolism in natural killing. Transmethylation and phospholipase A_2 activity were both found to increase during NK cell-target cell interaction. This evidence was obtained in large part through the use of agents which inhibited transmethylation enzymes (3-deazaadenosine, 3-DZA) or phospholipase A_2 (chloroquine, quinacrine, corticosteroids, and Rosenthal's inhibitor). Measurements of the release of radiolabeled arachidonic acid from the cell and analysis of the total phospholipids remaining within, confirmed the involvemnt of these pathways by quantifying the end-products of these reactions. Using more refined techniques of thin-layer chromatography (TLC) with multiple solvents, data were obtained to show that phosphatidylcholine, mainly generated via the transmethylation pathway, served as the substrate for phospholipase A_2. Similar studies using purified mononononuclear cell fractions enriched for large granular lymphocytes (LGL), known to be responsible for NK actiivty, showed that these cells demonstrated the same properties of transmethylation and phospholipase A_2 activation. It will be important to determine whether both protease and phospholipase A_2 independently contribute to lysis by NK cells or, more likely, whether these enzymes have important, sequential effects. The possibility of a role for reactive oxygen species in NK activity was examined by analysis of the ability of human LGL to undergo an oxidative burst in response to a variety of stimuli. Entirely ngative results were obtained with LGL, whereas monocytes had expectedly high levels of production. Thus this potential mechanism for cytotoxicity does not seem to be involved in NK activity.

IFN has been shown to have a variety of effects on immune reactivity, including the ability to rapidly augment cell-mediated cytotoxic responses such as the reactivity of NK cells, and macrophages, or monocytes. Some of these effector mechanisms may have in vivo importance in the resistance against tumor growth or against infections by various microbial agents. To obtain better insight into the nature of the diversity of such effects by IFN, various preparations of human leukocyte IFNs (12 purified or partially purified species of leukocyte, α, IFN) were tested for ability to augment the reactivity of NK cells and monocytes. At higher doses of IFN (i.e. > 500 units) all of the IFN species demonstrated significant augmentation of both NK activity and of monocyte-mediated cytolysis and cytostasis. However, if low levels of IFN were imployed (10-50 units) appreciable differences among the various pure species of human leukocyte IFN were seen. Generally, a similar pattern of relative potency was seen for the cytolytic activity by both monocytes and NK cells. However, comparison of the cytolysis and cytostatic assays demonstrated some very interesting differences. For example, IFN leukocyte $\gamma 1$, $\beta 2$ and $\beta 3$ species strongly induced augmentation of NK and monocyte-mediated cytolysis, whereas these preparations had low potency for augmentation of cytostatic activity. Conversely, leukocyte $\beta 3$ IFN generally a weak augmentor in the cytolytic assays but a high level augmentor in the cytostatic assay. Collectively these results demonstrated substantial quantitative differences in the ability of the various species of human leukocyte IFN to significantly augment levels of cell-mediated functions. Such results should have a significant impact in choosing the IFN species for appropriate clinical trials.

In addition to natural leukocyte IFNs, we have examined the newly available bacterial-produced homogeneous leukocyte IFN for its augmentation of cytotoxic activity of NK cells and monocytes. As with the purified species of leukocyte IFN, its relative potency was tested at various antiviral units. In parallel experiments, recombinant IFN and natural IFN were found to have similar

activities, with 10-100 units of IFN producing similar levels of augmentation. In our experiments the species specificity of recombinant IFN was maintained with no augmentation of mouse NK or monocyte functions. This demonstration that homogeneous recombinant IFN has potent effects on effector mechanisms provides a further basis for optimism regarding its potential usefulness in clinical trials.

Detailed analysis of treatment of human LGL with IFN, which causes augmentation of NK activity, has indicated that IFN has multiple effects on NK cells depending on the target cell tested. The effects include 1) an increase in the rate of the reaction, 2) an increase in the recycling time of the NK cells, 3) an increase in the number of lytic binders present in the population, 4) an increase in the number of binders against certain NK targets (especially seen with anchorage-dependent target cells), and 5) possibly a combination of all of the above. By using a single cell agarose assay, virtually all of the human LGL which bind to NK-susceptible targets in the presence of IFN are capable of killing their target, especially when the assay time is extended to between 4-24 hours. Similarly in a single cell cytotoxicity assay, IFN pretreatment of rat LGL was shown to have several effects, increasing: 1) the percentage of LGL which form conjugates with target cells, 2) the percentage of conjugate-forming cells which kill, and 3) the kinetics of lysis. The effects of IFN on one or another of these parameters varied with the target cell tested.

Since IFN has been shown to have wide-reaching effects on various immune functions, including boosting of NK activity, its effect on phospholipid metabolism was examined. IFN was found to increase endogenous phospholipase A_2 activity, as measured by release of prelabeled arachidonic acid, in peripheral blood mononuclear cells, monocytes, purified T cells and LGL. Paradoxically, this effect was least prominent in LGL. In contrast to the effect observed during NK-target cell interaction, IF did not change the total amount of arachidonic-acid containing lipids. Fractionation of labeled lipids by thin-layer chromatography of peripheral blood mononuclear cells showed a redistribution of unsaturated fatty acids, with decrease of labeled phosphatidylcholine concomitant with an increase in labeled neutral lipids. In experiments in which the effect of IFN on the transmethylation pathway was examined, decreased incorporation of tritiated methionine into phosphatidylcholine was observed. This provided support for the hypothesis that IFN increased phospholipase A_2 activity principally on substrate derived from the transmethylation pathway.

Several regulatory mechanisms have been identified which can depress the levels of NK activity. We have recently examined in detail suppression of NK activity by macrophages. Adherent peritoneal macrophages, induced by thioglycollate or obtained from untreated mice, suppress NK activity when incubated with these effector cells overnight at 37°C. These macrophages do not suppress at the effector phase since addition of macrophages to the cytotoxicity assay had no effect on NK activity. Macrophages from normal mice of several strains, including nude mice, were shown to have suppressor activity. In contrast, macrophages that have been activated *in vivo* with *C. parvum* did not suppress NK activity. Both spontaneous and IFN-boosted NK activity were inhibited by the addition of as few as 10% macrophages. Since there is low NK activity in the peritoneal cavity, this may represent one mechanism by which NK activity is naturally suppressed.

Another type of negative regulation was found in studies of short-term incubation of human peripheral blood mononuclear cells. After incubation for 1-2 hours in medium lacking human serum, increased NK activity was seen. This seemed

to be due to release from inhibition by human serum factors, since incubation in autologous or allogeneic serum prevented the augmentation and at high concentrations produced inhibition of activity. The serum-mediated effect appeared attributable to the degree of binding of cytophilic monomeric IgG to the cells. The overall pattern of results suggested that this mechanism may be involved in negative regulation of NK activity in vivo.

There have been some suggestions that NK cells can react against tumor cells not only by cytotoxicity but also by production of IFN. This possibility, of IFN production by NK cells, has been studied in detail by incubating highly enriched populations of human or rat LGL in vitro with tumor cells or other stimuli and measuring the levels of IFN in the culture supernatants. After overnight incubation, LGL, but not typical T cells, produced appreciable levels of IFN in response to NK-susceptible tumor cell lines and also to viruses (influenza and herpes simplex), poly I:C, BCG, Corynebacterium parvum, and to mitogens (PHA, Con A, Staph enterotoxin). Thus NK cells appear to have the capacity for self-regulation, being able to respond to certain stimuli by producing IFNs that in turn can agument their reactivity. These observations also indicate that NK cells may have a broader range of biologic effects than were initially appreciated, with a potential for directly producing an antiviral protein and for affecting the activity of other immune effector activities that are responsive to IFN.

We have continued to assess in vivo NK activity by measurement of the rapid elimination from the lungs of intravenously inoculated radiolabeled tumor cell lines. We previously showed that intravenous transfer of normal spleen cells or bone marrow cells could restore, at least in part, the depressed in vivo resistance induced by cyclophosphamide. We have now shown that the spleen cells required for this in vivo transfer of resistance have the characteristics of NK cells, being nonadherent and bearing the NK cell-associated antigen, asialo GM1. Using lethal X-irradiation as another procedure to depress in vivo NK reactivity in rats as well as in mice, we have found that transfer of spleen cells was also able to restore resistance.

These protocols have provided a good system for analyzing for the first time the possible in vivo effects of human NK cells. After depression of NK activity in nude mice, pruified populations of human LGL, which have been shown to be highly enriched for NK activity, were transferred and the mice were then challenged with radiolabeled human or mouse tumor cells. Such transfer of human LGL was able to increase the clearance from the lungs of the human NK-sensitive tumor cells, but had no effect on clearance of mouse tumor cells that are resistant to human NK activity.

Similar studies have been performed with transfer of mouse and human cultured lymphoid cells (CLC) that have NK-like cytotoxic reactivity. Such mouse or human CLC, when admixed with NK-sensitive tumor target cells and inoculated subcutaneously, reduced the survival of the tumor cells, as measured by local retention of radiolabel in our isotopic footpad assay, or by the incidence and rate of growth of detectable tumors. Systemic transfer of CLC had less impressive anti-tumor effects, perhaps because of problems with limited circulation or survival of the transferred cells, but some significant effects were observed. The results indicate some potential to the approach to immunotherapy by production and transfer of large numbers of cytotoxic CLC.

The in vivo radioisotopic assay has been used to assess the possible in vivo significance of in vitro detected suppressor cells for NK activity. This has been studied by two types of protocols: a) In the protocol of transfer of normal spleen cells to mice with treatment-induced depression of resistance to challenge with radiolabeled tumor cells, intravenous administration of adherent suppressor cells from low NK-reactive mice substantially interfered with reconstitution. b) Intravenous administration of adherent peritoneal cells with suppressor activity to normal, untreated mice, when injected simultaneously with, or a short time after, radiolabeled tumor cells, interfered with their clearance. Furthermore, in this latter protocol, the transfer of cells with suppressor activity could be shown to result in a higher number of detectable pulmonary metastases.

To begin to obtain direct evidence for a possible role of NK cells in in vivo resistance to primary tumors, we have recently initiated experiments with radiolabeled primary C3H mammary tumor cells. As with NK-susceptible tumor cell lines, inoculation of such primary cells into mice which varied in their levels of NK activity resulted in a parallel difference in the degree of clearance from the lungs of syngeneic mice. Thus, although the primary mammary tumor cells have only low levels of susceptibility to lysis of NK cells in vitro, this appears sufficient to play a role in vivo.

As one model to evaluate the possible role of NK cells in primary carcinogenesis in mice, we have injected urethane into young mice of various strains. Only some strains, particularly A/J mice, developed lung tumors after urethane treatment. In these susceptible mice, urethane also was found to cause an early, profound depression in NK activity. In contrast, urethane treatment of strains of mice that were resistant to pulmonary carcinogenesis did not cause detectable depression of NK activity. Administration during the latent period of normal bone marrow cells, according to a protocol known to reconstitute NK activity, could interfere with the subsequent development of lung tumors in A strain mice. Thus, it seems likely that depression of NK activity is one of the necessary effects of urethane in order to achieve detectable pulmonary tumorigenesis.

As a second model system for immune surveillance, C57BL/6 mice were treated with a schedule of multiple, low doses of X-irradiation, which is known to be highly effective in inducing thymic lymphomas in this strain. Such treatment was found to result in a substantial deficit in NK activity. This depressed NK activity could be restored by transfer of normal bone marrow cells, a procedure which has been shown to interfere with radiation-induced leukemogenesis. In contrast, transfer of bone marrow from beige mice did not restore NK activity. The leukemogenic effects of X-irradiation in beige mice were compared with those in heterozygous littermates, which have normal NK activity. The rate of tumor development thus far has been appreciably more rapid in the NK-deficient beige mice. Thus, NK cells do appear to have some role in the surveillance against radiation-induced lymphomas.

II. Cell-Mediated Immunity Against Tumor Associated Antigens

As a model for studies of cell-mediated immunity against tumor associated antigens, detailed investigations have been performed with continued cultures of human T cells (CTC), maintained with T cell growth factor.

During this year, we were able to examine the cytotoxicity mediated by CTC from patients with solid tumors. The patients' T cells, purified by nylon column

passage, were cultured with single cell suspensions of the tumor for six days. The blasts were then separated and expanded with TCGF-containing conditioned media, and the help of feeder cells. In several instances the CTC from the mixed lymphocyte-tumor interaction were found to be much more cytotoxic against the autologous, cryopreserved, ^{51}Cr-labeled solid tumor target cells than were any of several control CTC. Because this pattern of reactivity was of considerable interest and might indicate anti-tumor reactivity in these CTC populations, attempts have recently begun to clone these effector cells by limiting dilution, in the presence of feeder layers and TCGF.

III. Role of Macrophages in Resistance Against Cancer

Macrophages have been found to have several possible roles in host defenses against cancer. They can have direct cytotoxic effects against tumor cells, they can act as accessory cells for the generation of immune responses, and they can act as suppressor cells. We have continued detailed studies on each of these macrophage functions.

Macrophages have been shown to kill most tumor cells with a varying degree of efficiency. The question of whether there is any specificity to this interaction was reexamined. Macrophages from several strains of mice were activated by various agents and tested for their cytolytic activity against a panel of tumor targets. Regardless of the donor strain of the effector cells or the method of activation, the pattern of reactivity was similar. When several macrophage-like cell lines were tested in a similar manner, some gave a pattern that was similar to normal activated macrophages while others were distinctly different. This may reflect the origin of the cell lines from different subpopulations of macrophages or may be due to cytotoxicity by devergent effector mechanisms.

Several monosaccharides were tested for their ability to inhibit macrophage-mediated cytotoxicity and for comparison, their effects were also tested on NK activity. Different patterns of inhibition were noted with the activated macrophages, depending on the target cells employed. Only N-acetylgalactosamine inhibited cytotoxicity against L5178y, while mannose and N-acetylgalactosamine reduced the activity against RL♂1. When this was compared to inhibition of NK activity, it was noted that mannose gave reproducible inhibition of NK activity while glucose and galactose also inhibited cytotoxicity but to a lesser extent. The sugar inhibition data also provide evidence, for the first time, that there is heterogeneity of recognition or lysis by macrophages.

We investigated the possibility that the functional and biochemical changes that occur during the activation of mouse macrophages were accompanied by modifications in their macromolecular synthesis. Proteose-peptone elicited, macrophages were treated <u>in vitro</u> with poly I:C and their activation was evaluated by measuring cytolytic activity and glucose oxidation. Eight hours after poly I:C treatment, the RNA synthesis of the macrophages began to decrease, and by 24 hours it was 30-40% of the untreated controls. Protein synthesis was also inhibited in poly I:C-treated macrophages but only after 20 hours. Despite the decrease in macromolecular synthesis, macrophages at 16 hours after treatment with poly I:C were found to be functionally activated, with high levels of cytotoxic activity against ^{51}Cr-labeled target cells and a 3- to 4-fold increase in the rate of glucose oxidation. Treatment of resident peritoneal macrophages with poly I:C led to the same pattern, a decrease in macromolecular synthesis and high cytolytic activity. To determine whether this alteration in macromolecular synthesis

detected in vitro was a more general phenomenon associated with activation, we compared the RNA and protein synthesis of resident peritoneal macrophages (R-MØ) and macrophages activated in vivo by C. parvum (CP-MØ). CP-MØ showed a markedly reduced rate of RNA synthesis, ranging between 20-40% of the R-MØ. The protein synthesis, on the contrary, was not significantly different. We hypothesize that inhibition of macromolecular synthesis, particularly an early inhibition of RNA synthesis, is required for activation of macrophages.

Studies of inhibition of tumor growth by human peripheral blood monocytes were continued in a variety of assay systems. Growth inhibitory activity (GIA), measured by effects on tritiated thymidine incorporation, was found to be increased after pretreatment of effector cells with phorbol ester or IFN. Other substances, known to influence other monocyte or macrophage functions, such as prostaglandins, indomethacin, and corticosteroids, were without effect. Known inhibtors of superoxide or hydrogen peroxide generation had no effect on this assay in the dose ranges tested.

A new microassay system for the human GIA, which allows drastic reduction in the number of cells required for its performance, has been validated in terms of reproducibility and statistical norms. Using cryopreserved effector cells as well as cryopreserved target cells has permitted minimization of day-to-day variation in the assay.

Human monocyte function was also assayed by measuring cytotoxicity against tumor cell lines by a variety of isotope release assys. Using either ^{51}Chromium or ^{111}Indium release, monocytes from normal donors had demonstrable cytotoxicity against K562 cells which could be inhibited by agents which interfere with phospholipid methylation, as had been demonstrated for NK cells.

Accessory function by human monocytes has been assessed during the last year by their ability to facilitate the production of TCGF by CTC. It has been of considerable interest that K-562, a cell line derived from a patient with chronic myeloid leukemia in blast crisis, was as effective as monocytes in their accessory function for TCGF production by CTC. A systematic study of the available myelomonocyte human cell lines revealed that U-937 also had a similar accessory function, whereas HL-60, KG-1, and the pre-B cell line NALM-1 did not. K-562 lacks HLA-D,DR antigens, yet is able to cooperate with CTC. This may indicate that antigens other than those commonly detected by anti-HLA-D, DR sera may be important for macrophage-T cell cooperation. We found that K-562 and U-937 produced moderate to high levels of interleukin -1 (IL-1) upon stimulation with phorbol ester. The material from these cell lines may be suitable for extensive biochemical characterization, which has not been readily possible with material derived from blood monocytes. The availablility of two cell lines that produce IL-1, and cooperate with T cells, inducing their TCGF production, may represent an easy approach towards the analysis of the requirements for cell surface structures and mediators that are involved in such cooperation.

The ability of different populations of mouse macrophages to suppress the production of macrophage activating factor (MAF) by stimulated T-lymphocytes was investigated. We found that activated macrophages, infiltrating MSV-induced regressing tumors or recovered from the peritoneum of mice-injected with C. parvum, were able to actively suppress the production of MAF. MAF production by antigen-stimulated MSV-immune or alloimmune spleen cells and by normal spleen cells stimulated by Con A was susceptible to macrophage-dependent suppression to a

similar extent. In contrast, resident macrophages or those elicited by light mineral oil or proteose-peptone did not affect MAF production. While suppressor cells added at the time of the lymphocyte stimulation inhibited MAF production, the same macrophages added 4 hr after stimulation were ineffective. Therefore, it seems that the macrophages suppressed the early events of lymphocyte activation leading to MAF production.

To account for these diverse functions of macrophages, it seems likely that there are different subpopulations of macrophages, which vary in their stage of activation or differentiation. We initiated experiments to develop monoclonal antibodies against cell surface antigens on mouse and human macrophages in an attempt to provide reagents that could identify subpopulation of macrophages. Hybridomas were produced by the fusion of the mouse myeloma, NSI, with spleen cells from rats immunized with a macrophage cell line, $P388D_1$, or with bone marrow cultured macrophages that had been activated with poly I:C, and clones were selected that appeared to be producing antibodies specific for macrophages and further characterization was performed. One clone, 32.50, has been partially characterized. This antibody reacted with a variety of macrophage cell lines and also with some other types of tumor lines. However, the pattern of reactivity with normal cells was restricted to macrophages, polymorphonuclear leukocytes, and some bone marrow cells. This antibody, in the presence of complement, would also lyse a substantial portion of pyran- or C. parvum-activated macrophages but did not eliminate the cytolytic activity of these cells. More recently we have produced two other monoclonal antibodies that appear to be macrophage-specific. They do not react with normal T or B lymphocytes nor with non-macrophage tumor lines. However, they do exhibit high levels of reactivity against normal peritoneal macrophages, bone marrow macrophages and some macrophage cell lines. Further characterization is in progress to determine the range of reactivity of these antibodies and to determine whether they recognize subpopulations of macrophages. Similar attempts are in progress to produce monoclonal antibodies to subpopulations of human monocytes.

IV. Immunochemical Studies of Human Tumor Associated Antigens

Work on the purification of a human lung tumor-associated antigen (hLTAA) which is highly organ site restricted has been completed. Purification was accomplished from a saline extract of a human small cell lung carcinoma. The purification was facilitated by monitoring each step for hLTAA by single radial immunodiffusion assays which were developed here. In addition to previously reported steps of ion-exchange, G-200 sieving and antibody affinity chromatography, we employed preparative polyacrylamide gel electrophoresis (PAGE) and sieving high performance liquid chromatography (HPLC) to obtain the hLTAA in apparently homogeneous form. The hLTAA, designated LT-120, was found in the extract in two molecular sizes: 150,000 and 80,000 daltons. Sodium dodecyl sulfate PAGE of Bolton-Hunter ^{125}I-labeled antigen run under reducing conditions gave a major peak at 80,000 and much smaller peaks at 50,000 and 30,000 daltons. The antigen exists as two charge isomeric forms which differ in terms of isoelectric points (4.7 and 3.2).

A radioimmunoassay using the ^{125}I-labeled 150,000 dalton form has given early indication of clearly discriminating lung cancer patients' sera from those of normal, healthy individuals. Specifically, in a small group of samples we found 15 normal sera had a mean of 17 $\pm$ 22 ng/ml with none greater than 83 ng/ml (+ 3 S.D.) whereas 13 lung cancer patients with Stage I disease had a mean of 187 $\pm$

2.9 ng/ml with 7/13 greater than 83 ng/ml, and 15 lung cancer patients with Stage III disease had a mean of 277 ± 252 ng/ml with 12/15 greater than 83 ng/ml. We are in the process of identifying the nature of the serum inhibitor and will soon begin quantitating antigen levels in a large number of sera in order to assess the clinical usefulness of the assay.

Recent examination of concentrated urines from patients with lung cancer has indicated the presence of material which is immunologically related to LT-120 antigens. The antigen, as defined by precipitating reactivity in a radial diffusion assay, was also found in the urine of patients with other malignancies (melanoma, multiple myeloma, mycosis fungoides). In order to establish an assay system which would not involve concentration of large quantities of urine, a solid phase enzyme immunoassay (ELISA) was developed using patient's urine as inhibitor. The results correlated well with the antigen levels established by radial diffusion, but the new assay was much more sensitive (antigen levels of 50 ng/ml gave significant binding inhibition). Five normal urines used as controls gave no inhibition. In order to establish the molecular weight of the urinary inhibitor, seiving HPLC was performed and the fractions tested as inhibitors in the ELISA. Strongest inhibition occurred in the 50,000-80,000 dalton range.

V. Application of Immunological Procedures to Clinical Problems of Cancer Patients

One of the primary objective of LID is to develop and evaluate tests for their possible application to clinical problems of cancer patients. Therefore a laboratory has been set up for detailed immunologic monitoring of cancer patients. Procedures have been established to standardize and control the assays and to minimize various sources of technical variations in results. A major emphasis has been placed on the use of cryopreserved cells.

The following series of assays have been established for monitoring of immunologic reactivity of cancer patients: a) NK activity; b) monocyte-mediated inhibition of growth of tumor target cells; c) lymphoproliferative (LP) responses to mitogens and in mixed leukocyte cultures; and d) enumeration of lymphoid cell subpopulations, according to morphologic and cell surface markers: this includes the determination of percentage of LGL, of cells forming rosettes with sheep erythrocytes, and of cells reacting with various monoclonal antibodies (OKT3, OKT4, OKT8, OKT10, OKM1, anti-Ia) as measured with the fluorescein-activated cell sorter.

To be able to consistently have sufficient lymphoid cells from cancer patients for the measurement of multiple immunologic parameters, and to obtain dose response and other detailed information, procedures have been developed for miniaturization of some of the assays. It has been possible to adopt the procedures for performance of LP assays and of growth inhibition assays to mini-wells, which utilize only one-tenth the volume of medium and number of cells as those used in more conventional micro-assays. The incubation of the cells in the mini-wells is performed with the test plates inverted, with all of the cellular interactions thereby taking place in hanging droplets. The results obtained in such assays have been highly correlated with those obtained in parallel tests performed under conventional conditions, and therefore the patient specimens are now being routinely tested by the new procedure.

Recently much attention has been focused on the possible therapeutic efficacy of IFN. However, there is little information as to whether such effects may be mediated by direct anti-tumor effects of IFN or by the ability of this agent to augment host resistance. In view of the clear demonstration that IFN can substantially boost NK activity, one major hypothesis is that this is the mechanism for the clinical benefits from IFN. Alternatively, the effects of IFN on monocyte-mediated cytotoxic activity or other immunologic functions might be important. It therefore is necessary to determine the optimal doses and schedules of IFN administration for alteration of each of these parameters. Such an effort has recently begun with the highly purified recombinant leukocyte IFN A preparation of Hoffmann-La Roche. Patients with advanced cancer are being given a fixed dose of IFN, ranging from 1 million units to over 100 million units, in two divided daily injections or three times per week, for 28 days. These patients are being carefully monitored for the effects of the treatment in the range of immunologic parameters listed above.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08523-05 LID

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Biochemical and Serological Studies of Tumor-Associated Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.A. Braatz	Expert Consultant	LID	NCI
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COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Immunodiagnosis

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have purified a human lung tumor-associated antigen (hLTAA) to apparent homogeneity from an extract of a primary small cell carcinoma. The antigen, which was originally detected by double diffusion analysis in 84/98 lung tumor extracts, but not in normal tissues or other tumors, is a protein which exhibits size and charge heterogeneity. The protein has been characterized in terms of its sedimentation and diffusion coefficients, subunit size, isoelectric point, immunochemical reactivity with various antisera, and was shown to be distinct from α_1-antichymotrypsin, a normal protein which cross-reacts with another lung tumor-associated antigen we have studied. A radioimmunoassay (RIA) has been developed despite considerable difficulties encountered in radioiodinating the antigen. This RIA clearly distinguishes normal from lung cancer sera. The antigen is being purified from a human lung cell line which will be used as a constant source of antigen. A similar reactivity was also identified in the urine of certain patients with malignancies. Monoclonal and polyclonal antibodies are being produced against purified hLTAA. An assay for mouse MAF has been developed which measures plasminogen-dependent fibrinolysis and will be used in the purification and characterization of this lymphokine.

Project Description

Objectives: The objectives of this project are: 1) purify and characterize human tumor-associated antigens (hLTAA), 2) compare purified antigens in terms of their physicochemical properties and their structural and immunochemical relationships, 3) develop radioimmunoassays (RIA) for quantitating these antigens, 4) assess the usefulness of the RIA in the diagnosis of lung cancer and for monitoring the course of disease, 5) study the biological role of these antigens in the tumor, 6) develop monoclonal antibodies against the purified antigens which would be useful in Objectives 1-5, 7) isolate and characterize lymphokines that activate and/or inhibit the migration of macrophages and 8) produce, characterize and maintain a T cell hybridoma which would be a constant source of lymphokines for Objective 7.

Methods Employed: Radioiodination of purified protein antigens. Radioimmunoassays using the purified, trace-labeled protein antigens. Standard biochemical isolation and analytical techniques including gel filtration; ion-exchange chromatography; affinity chromatography; acrylamide gel electrophoresis; isoelectric focusing; ultraviolet, visible and fluorescence spectroscopy; ultracentrifugation; lyophilization; etc. High performance liquid chromatography (HPLC) in both the size exclusion and reverse phase modes. Autoradiography. Immunochemical techniques such as double diffusion in gel, radial immunodiffusion and immunoelectrophoresis. Techniques related to cell hybridization studies directed at the hybridization of murine lymphoid cells and long term maintenance of hybrid cells in culture. Monoclonal antibody production by B cell hybridomas and detection by enzyme-linked immunosorbent assays (ELISA) and RIA. Detection of products of hybrid and activated cells using assays for elastase, plasminogen activator, DNA, and factors B and C2. Agarose droplet technique for migration inhibition factor (MIF). In vitro activation of cytotoxic macrophages. Computerized analysis and graphic representation of RIA and other results using personal programs as well as public programs, such as MLAB.

Major Findings

I. Studies on a Human Lung Tumor-Associated Antigen

Work on the purification of a human lung tumor-associated antigen (hLTAA) which is highly organ site restricted and unrelated to α_1-antichymotrypsin (ACT) has been completed. Purification was accomplished from a saline extract of a human small cell lung carcinoma. The purification was facilitated by monitoring each step for both hLTAA and ACT using single radial immunodiffusion assays which were developed here. In addition to previously reported steps of ion-exchange, G-200 sieving and antibody affinity chromatography, we employed preparative polyacrylamide gel electrophoresis (PAGE) and sieving high performance liquid chromatography (HPLC) to obtain the hLTAA in apparently homogeneous form. The hLTAA, designated LT-120, was found in the extract in two molecular sizes: 150,000 and 80,000 daltons. Sodium dodecyl sulfate PAGE of Bolton-Hunter ^{125}I-labeled antigen run under reducing conditions gave a major peak at 80,000 and much smaller peaks at 50,000 and 30,000 daltons. Additional characterization studies have defined the following properties of the antigen: The antigen exists as two charge isomeric forms which differ in terms of isoelectric points (4.7 and 3.2) but are indistinguishable with respect to antibody reactivity, diffusion coefficients

($D_{20,w}$ = 4.3 X $10^{7}cm^{2}s^{-1}$), and sedimentation coefficients ($s_{20,w}$ = 4.6S). Although the antigen can be radioiodinated using Bolton-Hunter reagent, it does not iodinate with chloramine-T, lactoperoxidase or diazotized iodosulfanilic acid, suggesting absence of accessible tyrosine residues. Amino acid analyses will be performed to clarify this point.

A RIA using the ^{125}I-labeled 150,000 dalton form has given early indication of clearly discriminating lung cancer patients' sera from those of normal, healthy individuals. Specifically, in a small group of samples we found 15 normal sera had a mean of 17 $\pm$ 22 ng/ml with none greater than 83 ng/ml (+ 3 S.D.) whereas 13 lung cancer patients with Stage I disease had a mean of 187 $\pm$ 2.9 ng/ml with 7/13 greater than 83 ng/ml, and 15 lung cancer patients with Stage III disease had a mean of 277 $\pm$ 252 ng/ml with 12/15 greater than 83 ng/ml. We are in the process of identifying the nature of the serum inhibitor and will soon begin quantitating antigen levels in a large number of sera in order to assess the clinical usefulness of the assay.

The homogeneous preparation of the LT-120 antigen has been used to immunize a goat. Antiserum from that animal will be vigorously examined for titer, specificity, and possible use in an improved version of the RIA. Current results indicate that antibodies to the antigen are being produced but the titers of these early bleeds are still somewhat low. This antiserum shows specificity in that reactivity by double diffusion analysis can be demonstrated with two distinct lung tumor extracts, but not with a pooled extract of normal lung tissue. The animal will be boosted in an attempt to increase the titer. This second generation antiserum should prove to be very useful in our ongoing studies of this antigen. For example, the large quantities of serum already collected by plasmapheresis will provide substantial amounts of antibody which will be used as an affinity immunoadsorbent for routine antigen isolation. Our finite supply of first generation rabbit antisera would not have lasted for long if used for this purpose. In addition, this second generation antisera will be used in conjunction with the first antisera to develop a double antibody ELISA for quantitating the hLTAA. This would be based on attachment of antibody to the surface of a plate instead of attaching antigen, which is technically more difficult and uneconomical. Further, if the goat antisera develops antibodies with greater affinities, it could be used to develop a more sensitive RIA and, since the antisera will be plentiful, can readily be shared with workers in other laboratories.

Long term established cell culture lines derived from human lung carcinomas have been examined for the presence of this antigen. Seven of the eight lines extracted contained an antigen which was immunologically indistinguishable from the LT-120 antigen. Partial purification of this antigen from one of these cell lines (CHAGO) which was grown in roller bottle cultures, has been accomplished using procedures similar to those used to purify the LT-120 antigen. Thus antigenic activity was isolated from the cells by saline extraction and purified using HPLC size exclusion chromatography, DEAE-cellulose, affinity chromatography, and polyacrylamide gel electrophoresis. The bulk of the immunoreactive material has a relatively high isoelectric point since it did not bind to DEAE-cellulose at pH 8.1 and had a low electrophoretic mobility at pH 8.2. The size, by HPLC sieving, was between 50,000 and 80,000 daltons. This purification of the CHAGO antigen is being optimized for speed and yield in order to have an adequate and constant supply of the antigen.

In the past fiscal year we have initiated attempts at raising monoclonal antibodies (McAb) against the hLTAA. Reagent antibodies of varying and defined affinities and specificities would be of enormous value to the overall goals of this project. To this end, BALB/c mice were immunized by i.p. injection of purified, but not homogeneous, hLTAA, and then boosted with our purest preparation of antigen. The last injection was given i.v., three days prior to harvesting of the spleens. Fusions were performed according to standard protocols using X63Ag8 as the parent lymphoma. Growth was detected macroscopically in 90% of the wells after 5-10 days and the supernatants were tested for McAb production using an ELISA. The basis for the ELISA is as follows: partially purified hLTAA is bound to the surface of a microtiter plate, supernatants from growing colonies are incubated in the wells, washed, then incubated with a second antibody which binds to mouse IgG heavy and light chains and has covalently attached β-galactosidase. After washing, the plate is incubated with substrate and the positive wells identified. As a control, a similar ELISA is performed using a pooled normal lung extract (PNLE) in place of the hLTAA preparation. Hybridomas reacting with the PNLE or with the PNLE as well as the hLTAA were discarded. Those hybridomas showing specificity for the hLTAA alone were cloned by limiting dilution on feeder layers of macrophages. These supernatants were then again tested for immunoreactivity against hLTAA and PNLE in the ELISA. The hLTAA-specific hybridomas were then tested for reactivity with a homogeneous preparation of radiolabeled hLTAA in a RIA. Positive clones are then recloned and will be grown in BALB/c mice in order to enhance immunoglobulin production. To date we have obtained 102 hybridomas, out of 4500 colonies plated, which produce antibodies reactive with the hLTAA, but not PNLE in the ELISA. These are being further tested in the RIA for reactivity with the hLTAA.

Recent examination of concentrated urines from patients with lung cancer has indicated the presence of material which is immunologically related to the CHAGO and LT-120 antigens. The antigen, as defined by precipitating reactivity in a radial diffusion assay, was also found in the urine of patients with other malignancies (melanoma, multiple myeloma, mycosis fungoides). In order to establish an assay system which would not involve concentration of large quantities of urine, a solid phase ELISA similar to that described above was developed using patient's urine as inhibitor. The results correlated well with the antigen levels established by radial diffusion, but the new assay was much more sensitive (antigen levels of 50 ng/ml gave significant binding inhibition). Five normal urines used as controls gave no inhibition. In order to establish the molecular weight of the urinary inhibitor, seiving HPLC was performed and the fractions tested as inhibitors in the ELISA. Strongest inhibition occurred in the 50,000-80,000 dalton range. In addition, 7% polyacrylamide gel electrophoresis and antigen localization by Ouchterlony double diffusion indicated a very low mobility for the antigen (R_f = 0-0.08), similar to the CHAGO antigen. Urine from lung cancer patients will be collected as a potential source of large amounts of easily purifiable antigen.

II. Studies on Murine Lymphokines

During this past year our emphasis in this area was shifted slightly, to investigate in greater detail, assays for macrophage activation and their relation to the more conventional cytotoxicity assays which are the currently accepted definition of macrophage activation. Initial studies using ^{125}I-α-elastin

revealed numerous technical problems in attaching the substrate to a tissue culture plate while relatively fewer problems were associated with ^{125}I-fibrin. For this reason plasminogen activator (PA) production by macrophages has been chiefly studied. Using this assay for PA, which utilizes macrophages plated directly onto the ^{125}I-fibrin substrate, we have made a series of interesting and potentially useful observations: 1) Plasminogen-dependent fibrinolysis is an excellent assay for products of activated lymphocytes when run under a specific set of conditions, viz a) absence of serum and its inhibitors, b) defined input of plasminogen, and c) optimal cell concentrations of 5×10^5 - 1×10^6 cells/ml. Using direct attachment of macrophages to fibrin we have eliminated the need to concentrate supernatants in order to detect activity. In addition, the assay can be run within 48 hours after obtaining the macrophages. Recently we have adapted this method to microtiter plates. 2) We have compared secretion of PA from several macrophage cell lines with thioglycolate-induced peritoneal exudate cells (PEC) and found constitutive production of PA by the cell lines which was not affected by exogenous macrophage activation factor (MAF). Several of these lines produce large amounts of PA and could therefore be used as a source of enzyme for purification. An important secondary benefit to this study was the discovery that an appreciable portion of the PA in thioglycolate-induced macrophages is cell associated and would not be detected if supernatants alone were measured. Also non-plasminogen-dependent proteolysis was minimal when compared to PA secretion. 3) In attempting to correlate this assay with the current cytotoxicity assay available in collaboration with Drs. Taramelli and Holden, we find that endotoxin, an effective activator of macrophage cytotoxicity, does not activate mouse macrophages to produce PA, and when added simultaneously with or preceding MAF, does not enhance PA activity over that seen with MAF alone. However, if macrophages are activated either in vivo (using thioglycolate) or in vitro (using MAF), endotoxin depresses PA activity when added to macrophage monolayers. Consistent with our earlier findings, endotoxin does not interfere with the activation of plasminogen or the degradation of fibrin by plasmin, and cannot account for the decrease we observe. We can now account for the diverse reports in the literature of the effects of endotoxin on PA activity, and that is that macrophages must be activated before they are susceptible to the inhibitory effects of endotoxin.

Our previously constructed T cell hybrid, which was to be used as a source of MAF for biochemical studies, has proven to be unstable after approximately 3 months. Attempts at recloning frozen stocks of the hybrid have failed to yield an active MAF-secreting line. Therefore, we have been attempting to isolate new hybrids, and the use of the PA assay for screening has greatly improved the speed and efficiency of selection as compared with our earlier procedures.

Significance to Biomedical Research

Studies on the hLTAA system are relevant to both clinical and basic science. The significance of the work directed at developing a clinically useful radioimmunoassay for the detection or monitoring of lung cancer is obvious.

Recent experiments directed at quantitating hLTAA levels in serum and urine using a variety of assays including RIA and ELISA have yielded promising results. The LT-120 antigen, unlike previous lung tumor antigens described by us which were cross-reactive with normal serum components, appears not to be present in significant quantities in the serum or urine of normal, healthy individuals. Elevated

levels found in both serum and urine of lung cancer patients suggest the usefulness of this assay for diagnosis and/or monitoring of lung cancer patients. The evaluation of this antigen as a general lung cancer marker will be performed using the assays described here on a large number of clinical samples, which will provide a better assessment of the clinical utility of these assays.

The work with the lymphokines is directed at obtaining an understanding of these substances at the molecular level. Many lymphokines have been identified in crude supernatants but very few have been purified and characterized. Isolation of purified MAF, one of the goals of this study, should lead to a better understanding of its relationship to migration inhibition factor and to its mechanism of action. The production of stable T cell hybrids could be a general source of a number of these factors for further study.

Proposed Course

During the next year we plan to continue these studies as follows:

I. hLTAA Studies

1. Our major emphasis will continue to be isolation and characterization of various forms of the hLTAA from primary tumors, cell lines, and the urine and serum of lung cancer patients. The molecular and immunochemical properties of these forms will be evaluated in order to better understand their relationship. Despite the variety of forms displayed by the hLTAA with respect to size and charge, we hope to gain some understanding of them by peptide mapping using HPLC reverse phase techniques. This is a powerful method for examining related proteins and should give us an indication of the amount of structural overlap among the various forms of the antigen.

2. The purification scheme for the CHAGO cell line antigen will be optimized for efficiency and speed, since we hope this line will be the source of antigen for future studies, particularly those related to evaluation of the RIA in clinical studies. Again the use of HPLC in multiple modes promises to be an effective means to this end.

3. The development of a clinically useful RIA for lung tumor antigens is progressing well despite the occurence of some difficulties. One of these difficulties is the fact that the antigen does not iodinate well using standard procedures which directly label tyrosine groups. It appears the antigen simply contains little or no tyrosine, a point we hope to clarify soon with amino acid compositional analysis. We hope to circumvent this problem in one of the following ways: a) Modification of the Bolton-Hunter labeling technique which has already proven promising. b) Examination of the other techniques which iodinate groups other than tyrosine. c) Development of an ELISA which uses unlabeled antigen directly attached to plastic plates, or an ELISA based on one antibody bound to the plate to which unlabeled antigen would intereact and be detected with a second antibody-enzyme conjugate.

4. A more detailed definition of the occurrence of hLTAA in various tissues and cell lines will be undertaken using the more sensitive assays (RIA and/or ELISA). Cell lines other than lung cancer lines are being obtained and will be

examined as will extracts of normal tissues and numerous malignant tissues. Reagents are being exchanged with other investigators working in this area in order to determine the relationship, in any, of the hLTAA to other antigens which are being described. In addition, we will be testing these extracts for the presence of ACT since we have already demonstrated this protein in several of the lung cell lines we have obtained. This is a very interesting observation and will be pursued since it is not known why ACT, normally a liver product, should be produced by malignant lung cells.

5. Work on the generation of additional antibodies, both polyclonal and monoclonal, will continue. Goat antisera to purified hLTAA has proven to be reactive in early bleeds and will be tested after subsequent immunizations for increased titers. Hybridomas will be produced and screened using the rapid and sensitive ELISA and RIA techniques. Monoclonal antibodies will be characterized in terms of the specificity and affinity for the antigen. These should prove useful in determining precisely the distribution of antigenic determinants among various histologic forms of lung cancer and, if detected, among tumors of other organs. Normal tissues would also be examined. In addition, low affinity antibodies would be useful in further antigen isolation whereas high affinity antibodies could render the RIA more sensitive and specific.

II. Lymphokine Studies

1. Production of T cell hybridomas will continue in an attempt to obtain a stable hybrid which produces MAF activity. Our primary goal remains the biochemical characterization and mechanism of action of MAF, as well as its relation to migration inhibitory factor. Our recent development of an assay for PA will facilitate selection of MAF-producing hybrids and has in fact already proven useful in this regard.

2. We will explore collaboration with other investigators who are producing T cell hybridomas for other purposes as an alternative or supplement to preparing our own. Descriptions of such hybrids are appearing with increasing frequency and we have already made provisions to test some of these for MAF production by PA stimulation.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08500-07 LID

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Specific Immune T Cell Reactivity to Tumor-Associated Antigens in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.D. Bonnard	Visiting Scientist	LID	NCI	
Others:	B.M. Vose	Visiting Scientist	LID	NCI	(1/81 - 6/30/81)
	N. Navarro	Visiting Fellow	LID	NCI	(until 6/30/81)
	J.M. Alvarez	Visiting Fellow	LID	NCI	
	M. Gramatzki	Visiting Fellow	LID	NCI	
	R.R. Zicht	Biologist	LID	NCI	
	J.R. Fruehauf	COSTEP	LID	NCI	(10/80 -12/31/80)

COOPERATING UNITS (if any)

M, NCI; Surg, NCI; PhPath, NCI; NIDR; Natl. Naval Med. Ctr.; Walter Reed Army Hosp.; George Washington Univ.; Georgetown Univ.; Christie Hosp., Manchester; Madrid Univ.; Rome Univ.

LAB/BRANCH

Laboratory of Immunodiagnosis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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6.75	5.25	1.50

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We studied human T cell activation and regulation in the system of continued cultures of T cells that respond to T cell growth factor (TCGF). T cells cooperate with monocytes and with B cells during lymphoproliferation to the lectin phytohemagglutinin. Monocytes and a few myeloid and monocytic leukemia cell lines function as assessory cells and produce interleukin 1 (IL-1). We studied the TCGF producing cells, and cells responding to TCGF, by means of anti-T cell sera, Percoll gradients, and the presence of receptors for the Fc portion of IgG (FcγR). Cultured T cells (CTC) are cytotoxic in antibody-dependent cell-mediated cytotoxicity, natural cytotoxicity and specific immune cytolysis. Lymphocytes from cancer patients were sensitized to their autologous tumor cells, expanded as CTC and found to react specifically in cytotoxicity and by proliferation to the original stimulating tumor cells.

PHS-6040
(Rev. 2-81)

Project Description

This group has concentrated on work with continued cultures of human T cells. These cultured T cells (CTC) have been derived from fresh peripheral mononuclear leukocytes (PBL) or from cultures of *in vitro* sensitized lymphocytes, with the aid of conditioned media from phytohemagglutinin (PHA)-stimulated PBL, which contain T-cell growth factor (TCGF). We seek to develop this technology to achieve one major, long-term goal: the development of cellular reagents specific for tumor-associated transplantation antigens (TATA) in man. To achieve this goal, it is necessary to first obtain more basic information on the cellular requirements, and the soluble factors, involved in T cell immune responses in man.

Our present priorities are mainly to dissect the heterogeneity of T cell subsets found in PBL and CTC, particularly as they relate to the mechanisms of T cell activation and regulation: 1) a study of T cell-monocyte and T cell-B cell collaborations during stimulation with lectins, in terms of the resulting proliferation, production of mediators, and cooperation between structures at the cell surfaces; 2) an examination of the TCGF-producing T cells (Tp) and the T cells responding to TCGF (Tr), with regard to their maintenance in cultures; also by means of heterologous and monoclonal antibodies to T cell subsets and other cell separation procedures; 3) a study of whether CTC contain cells bearing receptors for the Fc portion of immunoglobulin G ($Fc\gamma R$), that may be regulated by immune complexes, and may have regulatory functions on other T cells; 4) a study of whether CTC contain natural killer (NK) and killer (K) cells, raising the question of their contribution to cytotoxicity by CTC against human tumors; 5) attempts to clone CTC with one or more of the above characteristics. This detailed view on the heterogeneity and functions of cultured T cells will allow us to adequately vary the conditions of our *in vitro* cultures, to enhance or eliminate certain T cell subsets and their functions, when we start to generate specific immune T cells against human TATA.

Our feasibility study on the development of cellular reagents specific for TATA in man is as follows: 1) improve culture conditions for CTC such as to achieve better cell yields, longer cell survival, and allow cloning of effector CTC; 2) analyze the relative contribution of various types of cytotoxicity by CTC against tumor cell lines and fresh solid tumors; 3) utilize proliferative responses and ^{3}HTdR-incorporation as an alternate means to detect CTC sensitized against TATA.

Major Findings

I. Introduction

Because deficient responses to tumor antigens, as well as other antigens, could be due to the lack or malfunction of one of several cell types participating in the response, it is important to detect all the cells involved in responses to antigens, and to design new methodologies to assay their function individually. Our demonstration of a role for TCGF-producing cells (Tp); and cells responding to TCGF (Tr), and also for monocytes in TCGF production, has opened up the possibility to test the function of these cells and their secreted products, separately, by appropriate protocols. We will review experiments on the assessory role of

monocytes in Section II-1, and on the subsets of T lymphocytes that get involved in responses to antigens in Section II-2.

There has been considerable interest over the past few years in the role that immune complexes (IC) may play in enhancing tumor growth. In fact, several empirical protocols have been developed to attempt to remove, by plasmapheresis, immune complexes found in certain cancer patients. So far, there has been little in depth biological information to support these clinical trials. Very little is known about the influences that immune complexes exert on human T cell responses to antigens. One well-documented finding has been their ability to abolish the lymphoproliferative responses of isolated T cells bearing receptors for FcγR. We have continued to explore the suppressive effects of IC on T cell lymphoproliferation, as reported in Section II-3.

One of the earliest studies with CTC in this laboratory has been the attempt to demonstrate that FcγR-bearing NK and K cells could be grown with TCGF, thus supporting the T cell lineage of NK cells. It now appears that NK and K cells expressing FcγR of low affinity can be maintained as CTC, and that large granular lymphocytes (LGL), the morphological equivalent of the functional NK or K cells, can be grown with TCGF under certain conditions. For our purpose, the presence of NK or K cells in CTC is important, because attempts to obtain CTC as cellular reagents with specific cytotoxicity against TATA require that one analyze separately the NK cells and the classical immune T cells within CTC populations for their potential use as such reagents. These efforts are described under Section II-4.

One of the major problems with the development of unique cellular reagents against TATA in man, using the CTC technology, has been the limited expansion and life-span of these cultures. Human CTC ususally undergo a crisis at 6-8 weeks and die. In experiments starting the cloning from a single cell, this means that less than 10^7 cells can be generated by the time of the crisis in most instances, which is not enough for extensive testing. It is thought that improvement of the level and quality of TCGF production could alleviate these problems. Progress in this area, described in Section III-1, is also of practical importance in view of the high cost of the commercially available TCGF preparations.

We studied in detail the autologous mixed lymphocyte-tumor reaction (MLTR) in cancer patients. Thus, we investigated the effects of feeder layers and TCGF during primary and secondary stimulation, and looked both at cytotoxic effector cells and cells that would proliferate to the original stimulus, as described in Section III-2 and III-3 below. Using these techniques we expect to generate cellular reagents against TATA in man.

II. Biological Studies on T Cell Heterogeneity, Activation and Regulation.

1. Studies of T cell-monocyte and T cell-B cell collatorations during stimulation with lectins. We have demonstrated that Tp, whose fate was hitherto unknown, grow out with CTC. When CTC were adequately stimulated with PHA in the presence of purified monocytes as accessory cells, they produced TCGF in the supernatant, and they proliferated to this endogeneously-produced TCGF. It could be that Tp and Tr are the very same cells, and this has important implications for our model of T cells activation. Also, the possibility exists that Tp cells could

be cloned, thus providing monoclonal sources of TCGF. Examination of the activity of TCGF from different clones may provide evidence for heterogeneity of TCGF, a much debated question. The presence in CTC of the right kind of Tp cells may be important for the long-term survival of the culture.

As controls for the blood monocytes in these experiments, we used cells from a variety of cell lines, and most did not have the same accessory function. However, K-562, a cell line derived from a patient with chronic myeloid leukemia in blast crisis, was as effective as monocytes in their accessory function for TCGF production by CTC. A systematic study of the available myelomonocyte human cell lines revealed that U-937 also had a similar accessory function, whereas HL-60, KG-1, and the pre-B cell line NALM-1 did not. We find it of considerable interest that K-562 lacks HLA-D,DR antigens, yet is able to cooperate with CTC. This may indicate that antigens other than those commonly detected by anti-HLA-D, DR sera may be important for macrophage-T cell cooperation. The availability of monoclonal antibodies to HLA-D,DR subregions and to K-562 cells should enable us to make progress in this area. We found that K-562 and U-937 produced moderate to high levels of IL-1 upon stimulation with the tumor promoter phorbol myristate acetate (PMA). IL-1 in these supernatants were measured on C3H/HeJ mouse lymphocytes, in the presence of PHA. C3H/HeJ thymocytes did not respond to PHA alone, or to PMA alone. The material from these cell lines may be suitable for extensive biochemical characterization, which has not been readily possible with material derived from blood monocytes. The availablility of two cell lines that produce IL-1, and cooperate with T cells, inducing their TCGF production, may represent an easy approach towards the analysis of the requirements for cell surface structures and mediators that are involved in such cooperation. In particular, clones of K-562 or U-937, or hybrids made with K-562 or U-937, could be analyzed for their cell surface markers, their production of IL-1, and their ability to cooperate with CTC. Some of these results may shed light on the cooperative events between normal T cells and monocytes or macrophages during the immune response.

During our study of the K-562 cell line, known to represent precursor cells of the myeloid and erythroid series, we also examined human granulocytes for their ability to cooperate with CTC in our system. We found that our granulocyte preparations worked as well as monocytes, provided we used higher cell numbers. However, we are not sure, at this point, whether this activity is mediated by contaminating macrophages, some special activity of the contaminating erythrocytes, or the granulocytes themselves. This deserves further study.

Our new system with CTC stimulated with PHA and monocytes also represents a very practical test of monocyte accessory function, and could be applied to testing monocytes from patients with a variety of diseases.

We previously described the enhancement of TCGF production by PBL when cells from B lymphoblastoid cell lines (B-LCL) were added to the mixtures of PBL and PHA. Only B-LCL and not T lymphoblastoid cell lines had the effect, which excluded a role for HLA-A,B or C alloantigens. Furthermore, Daudi, a B-LCL lacking these antigens, enhanced well. HLA-D,DR antigens did not seem to be involved either, since an HLA-D,DR negative mutant cell line and its parental line, kindly provided by Dr. D. Pious, both enhanced to the same level. We are presently studying the possible roles of EBV antigens, FcγR, and of soluble products released specifically by the B-LCL. It will be important, also, to use

normal peripheral B cells to conclude whether this reaction indicates a T cell-B cell collaboration, that takes place normally.

2. Studies of T cell subsets, particularly Tp and Tr. According to our previous model of T cell-activation (Bonnard et al, J. Immunol., 123, 2704, 1979), the subset of Tp may be different from a subset of Tr, which grow as CTC. The experiments described above on the production of TCGF by CTC suggest, on the other hand, that Tp and Tr could be the same cells. Alternatively the possibility exists that CTC contain separate populations, Tp and Tr. This was studied further in collaboration with several members of LID. First, they looked at subsets of freshly isolated PBL for TCGF production. The recently developed technique of Percoll gradients allowed the separation of PBL into LGL, mostly NK cells, 50% of which form rosettes with SRBC at 4°C, and into small lymphocytes, mostly typical T cells. Further SRBC-rosetting of the LGL fraction at 29°C yielded greater than 95% pure LGL preparations. Examination of the TCGF production by LGL and T cells showed T cells to be much better producers than LGL. The LGL preparations produced low levels of TCGF, but it is unclear as yet whether this level of TCGF was due to contamination with minute numbers of T cells. In any event, this was extended by the demonstration that both highly purified LGL preparations, as well as highly purified T cells, grew on exogeneous TCGF. These results were not simply due to a failure to separate these cells properly, since LGL in culture kept their typical morphology, and characteristic pattern of cytotoxicity, quite distinct from that of cultured T cells, as described in more detail in report Z01 CB 08503-01 LID. Thus, both the LGL fractions and the T cell fractions contained Tr cells.

Leukemia T cells might also fall into the categories of Tp and Tr. Therefore, during the past year we examined the blasts of two patients with T cell malignancies for their ability to produce TCGF and to respond to exogeneous TCGF (Tr function). These results, however, were difficult to interpret due to potential contamination with normal PBL. Typically, when we were able to grow CTC from the cells of a patient with a monoclonal OKT8+ chronic T cell leukemia, after 14 days the CTC had the same cell surface markers as normal control CTC. However, with other T cell malignancies, it may be possible to grow the clone of malignant cells with TCGF, or to show that the cells produce TCGF. Therefore, this study is being pursued.

3. Study of CTC with an FcγR and their regulation by immune complexes. Several lines of evidence have indicated that at least a portion of the CTC derived from PBL were FcγR positive cells. First, we found that fluorescein-conjugated aggregated IgG stain these cells, as revealed by the fluorescence-activated cell-sorter. Second, many populations of CTC could mediate antibody-dependent cellular cytotoxicity (ADCC). CTC also were able to mediate NK, which in fresh PBL is mediated by FcγR positive LGL. Recently, it was found that a percentage of the CTC derived from PBL had the morphology of LGL. Yet, we were not able to detect CTC which formed rosettes with EA (complexes of ox erythrocytes and rabbit anti-ox erythrocyte IgG). Thus the FcγR on CTC must usually be of low affinity, not readily detectable with EA.

We next attempted to confirm the presence of FcγR on CTC indirectly, by the ability of IC to regulate lymphoproliferative responses of FcγR positive cells. Specifically, IC have been reported to abrogate the lymphoproliferative

responses to PHA of murine spleen cells, and of isolated FcγR positive human PBL T cells. Because, our new model of T cell activation suggests the following two steps in these responses, it could be that IC acted on 1) the production of TCGF by Tp cells or 2) the ability of Tr or CTC to respond to TCGF. In order to obtain possible insight into the regulation of proliferation by IC in vitro, we used IC made with various concentrations of IgG and mixed them in various concentrations with lymphocytes stimulated with PHA, TCGF, both, or nothing more than IC. IC, in particular EA that were tested extensively, did not appear to block the production of TCGF by PBL, by FcγR positive T cells, or by fractions enriched for LGL. On the contrary, preliminary experiments suggest that EA caused the production of TCGF by FcγR positive PBL and T cells. (As noted above, it is unclear so far whether LGL can produce TCGF). In contrast to those earlier results obtained with positively-selected FcγR-bearing cells, EA did not inhibit the lymphoproliferative responses to PHA of PBL, and, in fact, enhancement of proliferation was often observed when EA were present. Further experiments showed that the lymphoproliferative responses to PHA of fractions enriched for LGL or small T cells were augmented by a range of concentrations of EA. This suggested that the separation of LGL on Percoll, most of which had FcγR, was unable to reproduce findings with positvely-selected FcγR-bearing cells (inhibition).

In sum, these experiments clearly demonstrated that IC had no negative effects on TCGF production. The inhibitory effects of EA on T cell lympho-proliferative response to PHA was probably due to an early effect on the acquisition of the TCGF-receptor and the binding of TCGF. This effect seemed transient insofar as CTC lost their ability to bind EA and EA could no more suppress CTC's response to TCGF. However, CTC appeared still capable of binding soluble immune complexes, and these IC suppressed CTC's responses to TCGF. These experiments point toward a major immunoregulatory role of IC and low-affinity FcγR on T cells that were not readily detectable with the conventional EA-rosetting techniques. Thus, problems of regulation by IC, that have long been considered important in vivo, are being unraveled here by in vitro experiments using the regulation of T cell proliferation as an indicator of the presence and activity of low affinity FcγR. Furthermore, these results are consistent with our demonstration, last year, of ADCC activity in CTC.

4. Presence of NK and K cells in CTC and growth of NK and K cells on TCGF-containing supernatants. In a collaborative study within LID, others have demonstrated that highly pure LGL isolated by Percoll gradient from fresh PBL were able to grow as CTC. Alternatively, a small contamination of the LGL by small T cells with their rapid growth in culture could account for these results. One then wishes a cell-surface marker of NK and K cells, that would be maintained in culture longer than OKM-1, while the OKT-3 marker is acquired, thus shedding more light on the maturational steps of NK cells towards mature T cells. Efforts were made to raise monoclonal antibodies to purified human LGL in mice. Following several protocols to immunize mice with LGL, and studies on numerous colonies and clones of mouse-murine hybridomas, two clones seemed to hold some promise of specific reactivity with LGL. Clones were screened first for content in IgG or IgM, then for reactivity with LGL but not small T lymphocytes (using an ELISA assay), then for absence of reactivity with B lymphoblastoid cell lines. Further analyses of the clones' supernatants was conducted using a Staphylococcus aureus binding assay, a Protein-A-red blood cell rosetting technique, and analysis on the fluorescence-activated cell-sorter. Our results are only preliminary at this

time. Beyond the analysis of LGL in culture, the availability of a monoclonal antibody against LGL and the NK and K cells may be extremely useful in the dissection of different cytotoxic effector cells against human tumors found in CTC. Last year, we reported on the need to distinguish between NK cells and specically sensitized lymphocytes in these cultures. A monoclonal antibody which would fix complement may enable one to get rid of the component of cytotoxicity due to NK cells in these cultures, and be left with specifically sensitized CTC, e.g. against a human tumor.

III. Clinically Oriented Studies on Cellular Reagents That Might Detect Tumor-Associated Transplantation Antigens in Man

1. Improved culture conditions for CTC. We have continued to produce good quality TCGF preparations from lymphoid cells of tonsils and spleens. Of particular interest this year was our demonstration that excellent TCGF preparations were obtained from thoracic duct lymphocytes. Certain patients with prospective kidney graft undergo intense lymphoid cell depletion by thoracic duct drainage, and massive numbers of cells become available for TCGF production. Since these protocols are being performed all over the country, our new method may be generally applicable.

We have also made progress in the maintenance and long-term culture of CTC by eliminating infection with mycoplasma. Mycoplasma have been known for quite some time to be toxic to cell lines, and in particular to malignant T cell lines in culture. Our experience this year extended these findings to CTC which seem extremely susceptible to mycoplasma infection. This was noticed during our above studies on the potential accessory role of various cell lines for T cell proliferation to PHA. A control in these experiments include CTC and TCGF with the cell line. A number of cell lines proved to inhibit CTC responses to TCGF, and these results were attributable to infection with mycoplasma. Our finding is of considerable practical importance, because the commercial laboratories that prepare TCGF have not been aware of the deleterious role of mycoplasma. They will now be able to take specific steps to avoid such contamination.

2. Cytotoxicity mediated by CTC against autologous fresh and cryopreserved tumors. In order to get specific immune CTC against human tumors, once would have to distinguish these cells from effector cells which mediate other types of cytotoxicity (due to polyclonal activation, lectin-induced cytotoxicity, and NK and K cell activity).

During this year, we were able to examine the cytotoxicity mediated by CTC from patients with solid tumors. The patients' T cells, purified by nylon column passage, were cultured with single cell suspensions of the tumor for six days. The blasts were then separated and expanded with TCGF-containing conditioned media, and the help of feeder cells. In several instances the CTC from the MLTR were found to be much more cytotoxic against the autologous, cryopreserved, ^{51}Cr-labeled solid tumor target cells than were any of several control CTC. Because this pattern of reactivity was of considerable interest and might indicate anti-tumor reactivity in these CTC populations, attempts have recently begun to clone these effector cells by limiting dilution, in the presence of feeder layers and TCGF. As mentioned above, however, there are still considerable technical

problems with the maintenance of these clones in culture in order to reach sufficient cell numbers for more detailed studies.

3. Secondary proliferative response of CTC as assay for recognition of TATA. Using the CTC derived from autologous MLTR of patients with solid tumors, we have been able to demonstrate, on the first few experiments done, the capacity of the original stimulating tumor cells, and some but not all allogeneic tumor cells, to elicit a secondary proliferative response. The antigens responsible for such stimulation are under study: it will be necessary to distinguish recognition of TATA from responses to normal auto-antigens.

IV. Collaborative Projects on CTC with Other Investigators.

1. Studies on alloantigen-reactive chimpanzee CTC. We had initiated this collaboration with Dr. Strong, NMRI, NNMC, following our demonstration in man that lymphocytes sensitized to alloantigens and expanded on CTC could serve as excellent primed lymphocyte typing (PLT) reagents. A long-term aspect of this study has been the in vivo injection of autologous PLT in attempts to raise anti-idiotype sera. Our interest in this study was to learn more about the prospective risks of passive immunotherapy of cancer patents with CTC primed to their autologous tumor. This could possibly result in unwanted clinical effects, and in the production of anti-idiotype antibodies, with enhancement of tumor growth. It was very easy to raise specific PLT-CTC using chimpanzee lymphocytes, and two animals received several injections of up to 10^9 cells. All these injections were very well tolerated. One animal developed an anti-PHA antibody. There were no detectable anti-idiotype raised in this series of experiments. However, PLT-CTC of one chimpanzee proved excellent target cells for an anti-idiotype serum that had been raised several years ago. Because PLT-CTC can be expanded to large numbers with TCGF and can be radiolabeled in culture, this may now allow the immunoprecipitation of the idiotypic glycoprotein.

Significance for Biomedical Research and the Program of the Institute

The capability to generate large numbers of pure and functional T cells from any given individual or population of lymphocytes opens considerable avenues for use of these cells in in vitro assays of cell-mediated immunity. The technique may turn out to be the cleanest approach to the purification of considerable numbers of T cells reactive against human TATA, and this will certainly hold a very high priority in many cancer research laboratories for years to come. These studies are also potentially useful for the immunodiagnosis of cancer and in measuring changes of immunity in response to chemotherapy or immunotherapy. This would fall under Objective V, Approach 4 and Objective VI, Aproaches 1 and 4 of the National Cancer Plan.

Detection of tumor antigens and of immune reactions to human tumor-associated antigens is a major goal in tumor immunology. The development and standardization of in vitro assays for cell-mediated immunity is one of the most important aspects. Because immunogenicity cannot be studied directly in the human, the evaluation of immunologic reactivity and the antigenicity of tumor cells by in vitro assays is of paramount importance. This would fall under Objective V, Approach 4 of the National Cancer Plan.

The question of whether immunosuppressive factors play a role in interfering with effective immunity is also very important. It appears that the stimulation of the host immune system may be useless, unless one can first rid the body of the inhibitory phenomena related to tumor cell growth. Investigation in this area would fall under Objective IV, Approach 4 and Objective VI, Approaches 1, 2, and 4 of the National Cancer Plan.

The capability to distinguish between different T cell subsets and their functions would represent very significant progress towards the identification of T cells immune to tumor-associated antigens in cancer patients. This may lead to a better understanding of reactions against the tumor that may be important in vivo. The findings of our laboratory that T cells with various functions can be adequately studied in continued cultures open major avenues of research. Further, techniques are now available to study the factors that mediate the needed cellular collaborations during immune responses to tumors. It is possible that large quantities of T cells immune to the tumor or of factors that enhance responses to the tumor could be used in immunotherapy. This would fall under Objective V, Approach 4 and Objective VI, Approaches 1, 2, and 4 of the National Cancer Plan.

Proposed Course of Project

Extensive studies on cellular cooperation during T cell activation and regulation will be continued : a) T cell-macrophage collaboration during stimulation with PHA, Con A, lipopolysaccharide or soluble antigens in terms of resulting production of interleukin-1 (IL-1), TCGF, colony stimulating factor (CSF) and interferon (IFN). These soluble factors are all thought important in the circle of activation between T cells and macrophages. Phorbol myristate acetate (PMA) and monoclonal antibodies that are mitogenic for T cells will also be used as alternative stimuli. Monocytes and T cells have been reported to interact via Ia antigens in experimental models. We will also look at the role of HLA-D,DR structures, the homologue of Ia in the mouse, during TCGF production and activation of Tr. It is of considerable interest that K-562 cells collaborate well with T cells, yet seem to lack HLA-D,DR antigens. A possible explanation for this is that K-562 cells may bear a limited part of the HLA-D,DR complex, not detected in complement-dependent cytotoxicity with most anti-HLA-D,DR sera, but retaining the accessory role for PHA. It is very important to assess whether the K-562 determinant can also present soluble antigens to T cells. To assess the potential of further uses of K-562 for defining a human determinant involved in T cell-monocyte collaboration, we plan on using anti-HLA-D,DR monoclonals on both K-562 cells and blood monocytes, in attempts to block their accessory functions. Because this genetic determinant could be asociated with other cell surface antigens, we will also use monoclonal antibodies against K-562, monocytes, FcγR, and β2-microglobulin in this study. b) Further characterization of the subsets of T cells that get involved in cooperative events with monocytes, or with other T cells, during TCGF production and subsequent proliferation, by means of cell separation techniques and monitoring with monoclonal antibodies against T cells. Also, sequential analysis of CTC populations for cell surface markers that may reveal important shifts in the composition of the growing populations. Special emphasis will be placed on detection of constantly proliferating cells which are needed for long-term growth, and on monitoring the possible appearance of suppressor cells which might explain the death of many cultures around day 28. c) Comparison of the role of typical small T lymphocytes vs. LGL, and also FcγR negative vs. FcγR positive

lymphocytes in the regulatory circuitry. d) Further characterization of the accessory activity of the leukemic cell lines K-562 and U-937, for insight they may provide on the normal interactions between T cells and monocytes. These cell lines also might serve as useful feeder layers for CTC clones. They themselves could be cloned, or hybridized in collaborative studies, to analyze by somatic hybridization techniques, the cell surface structures and the metabolic products that are involved in their interaction with T cells. e) Elucidation of the mechanism of action of B lymphoblastoid cell lines in their boosting of TCGF production, and a study of whether normal B cells have the same effects. Studies will also be performed to determine whether B lymphoblastoid cell lines are useful as feeder layers for CTC clones, and may help overcome the crisis.

Efforts will be made to further examine the functional role of low affinity FcγR on T cells, upon IC binding. a) Attempts to confirm our hypothesis that EA inhibit the initial events on Tr during PHA-stimulation, but are unable to inhibit the later proliferation of CTC to TCGF. b) Demonstration that soluble IC may be more efficient than EA at the later stages, possibly due to higher level of binding to low affinity FcγR. c) More experiments to study the effects of interaction of FcγR positive T cells and IC on the production of lymphokines.

We will continue our efforts to develop cellular reagents against TATA in man. This will include: a) Attempts to maintain CTC in long-term cultures, and to clone CTC with maintenance of their cytotoxic or proliferative function. b) Analysis, by limiting dilution experiments, of the number of proliferating units which are precursors of the desired CTC. c) Mixed lymphocyte tumor reactions in human lung cancer and other solid tumors to examine cell growth with TCGF, and the development of specific immune T cell reactivity against tumors, namely cytotoxicity for, and the ability to be restimulated to proliferate by the original tumor cells. d) Examination of the specificity of these reactions, in particular whether they are directed against antigens present on normal cells (possibly Ia-like antigens), or against TATA, and whether they are HLA-restriced.

Overall, there is much evidence that our biological studies on T cell heterogeneity, activation and regulation have had considerable impact on our conceptual and methodological approach to the work with MLTR and the development of cellular reagents against TATA in man. We tend to favor these systematic studies over premature attempts to investigate cancer-related immunity. It is important, however, to remain alert as to possible developments that may directly apply to the immunodiagnosis or immunotherapy of cancer.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08503-01 LID

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cellular Immune Responses in Human Experimental Tumor Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human natural killer (NK) cells and K cells mediating antibody-dependent cellular cytotoxicity have been shown to be large granular lymphocytes (LGL). The majority of LGL form lytic conjugates with a large panel of NK-susceptible target cells. Interferon causes augmentation of NK and K cell activities of LGL and only LGL demonstrated either spontaneous or interferon activated NK activity. The mechanism of augmentation appears to be multiple: 1) increase in lytic efficiency, 2) increase in lytic binders, 3) increase in number of binders, and 4)increased recycling of effector cells. Analysis of the phenotype of LGL with monoclonal antibodies demonstrated sharing of a variety of antigens with both T cells and monocyte cells. Cultures of highly purified LGL in the presence of T cell growth factor demonstrated typical morphology and cytotoxic patterns of fresh NK cells.

Project Description

The object of this project is (1) to study the characteristics of the effector cells involved in natural cell-mediated immunity to tumors in man, 2) to study factors regulating the levels of activity of these effector cells, (3) to analyze the various roles of subpopulations of human lymphoid cells in the immune response, and the suppression of the immune response to tumor antigens, (4) to correlate these in vitro findings with in vivo clinical disease, to determine the relative importance of natural killer (NK) cells in tumor surveillance and in resistence against tumor growth.

Major Findings

I. Natural Cellular Immunity Against Tumor Cells

We have performed extensive studies in natural cell-mediated immunity against tumor cells. During the past year our studies have focused primarily on the following areas: a) characteristics of NK cells, b) specificity of NK cells, c) the factors and agents affecting the level of cytotoxic activity and, d) the cytotoxicity by cultured cells.

A. Characteristics of NK Cells

A major advance in the characterization of human NK cells has come from the finding of their close association with a subpopulation of lymphoid cells, termed large granular lymphocytes (LGL), that possess a distinct morphology with Giemsa staining containing azurophilic granules, with pale, blue-staining cytoplasm. A considerable portion of these LGL form lytic conjugates with NK-susceptible targets. These cells have been isolated from peripheral blood lymphocytes using discontinuous Percoll density gradients and subsequent further purification by rosette formation with sheep erythrocytes at 29°C, to minimize contamination with small typical T lymphocytes. Using these procedures, we have been able to reproducibly obtain fractions containing >90% LGL by morphological analysis and containing <1% mature T cells as determined by morphology and monoclonal antibody analysis. Virtually all of the LGL have Fc receptors for IgG and they possess most, if not all, of the NK cell activity against NK-susceptible targets. We have extended our previous results by examining in detail a large variety of NK-susceptible targets, including both lymphoid and anchorage-dependent lines. A high degree of correlation existed between the presence of LGL and NK cytolysis of this large panel of targets. Fractions which were devoid of LGL were also devoid of NK activity. Also, fractions which were devoid of LGL could not be induced to become cytotoxic by the addition of activating agents such as interferon or interferon-inducers. Detailed analysis of treatment of LGL with interferon, which causes augmentation of NK activity, has indicated that interferon has multiple effects on NK cells depending on the target cell tested. The effects include 1) the increase in the rate of the reaction, 2) an increase in the recycling time of the NK cells, 3) an increase in the number of lytic binders present in the population, 4) an increase in the number of binders against certain NK targets (especially seen with anchorage-dependent target cells), and 5) possibly a combination of all of the above. By using a single cell agarose assay, virtually all of the LGL which bind to NK-susceptible targets in the presence of interferon are capable of killing their target, especially when the assay time is

extended to between 4-24 hours. Coupling the purification and the single cell assay, we have estimated that >70% of the LGL are functionally active NK cells. However, a small proportion of the LGL, a least 10-15%, do not appear to be cytolytic cells and can not be induced with interferon to be cytolytic cells. It remains to be elucidated whether these cells possess other immunological functions or if they are either pre-NK cells or post-NK cells. We have also demonstrated LGL in human spleen cells and with slight modification of Percoll gradients have obtained highly enriched LGL fractions from human spleen. The presence of LGL morphology has been surveyed and found in most mammalian species including man, nonhuman primates, dogs, cats, sheep, rodents, cattle and sheep. The functional association with LGL morphology and NK cell function has been difficult in several species due to the lack of proper targets. However, this association has been seen with human, nonhuman primates, rat and mouse effector cells.

Because of the ability to obtain highly purified LGL fractions and their strong association with human NK cell activity, we were able to perform detailed phenotypic studies with these effector cells and compare them to the phenotype of typical small, mature T lymphocytes. This has been performed using a series of monoclonal antibodies which have been recently become available. This analysis was performed using fluorescence activated cell sorter which gives both the frequency of positive cells as well as the relative density of the antigen on the surface of the cells A considerable proportion of LGL reacted with antibodies OKM1 (60-80%) and anti-Ia (15-30%). 25% reacted with OKT8, which is similar to its expression on T cells. In contrast, however, very few LGL reacted with OKT3 or OKT4 antisera, which react with a high proportion of mature T lymphocytes. OKT11A and 9.6 antibodies, which react with the sheep erythrocyte receptor, also reacted with approximately 50% of the LGL. However, OKT10 (which has been found to react mainly on pro-or early thymocytes) reacted with a high proportion of LGL, whereas none of the other peripheral blood lymphoid cell populations were positive. It should be noted that the reactivity with OKM1 raises the question of some relationship of LGL to monocytes, but this antigen may be a differentiation antigen shared by a variety of cell lineages. In addition to the phenotypic analysis of LGL, a functional correlation of the various antigen positive or antigen negative cells was performed. By depletion of the cells on antibody monolayers against the mouse monoclonal antibodies, we were able to determine that most, if not all, of the active NK cells were OKT10 positive and the majority were also OKM1 positive. A significant proportion (approximately 1/2) of the LGL were E-rosette positive as measured by 9.6 or OKT11A. An insignificant proportion of the functional NK cells possessed the OKT8 or Ia antigens.

The ability to isolate and identify effector cells responsible for the NK activity has led us to examine the cytotoxic mechanisms involved in cytolysis. T cell cytotoxicity has been demonstrated to involve three major steps: 1) target cell binding, 2) programming for lysis and 3) cytolysis. Because of the ability to demonstrate and examine LGL target cell binding, we were able to examine a variety of agents and determine whether they effect either binding or cytolysis. We first examined a variety of enzymes which were shown previously to have some effects on NK activity. Both papain and trypsin severely diminished both cytotoxicity and the conjugate-forming ability of LGL. Conversely, lipase, which was previously demonstrated not to diminish the NK activity, showed no significant decrease in the ability of the cells to form conjugates or kill NK susceptible

targets. The analysis of a variety of metabolic agents, which included cholera toxin, prostaglandins, phorbol myristate acetate (PMA) and Staph protein A, demonstrated that while cholera toxin and prostaglandins diminished the cytotoxicity, these had no major effect on the binding of the effector-target cell. Conversely, PMA significantly depressed both the binding and the cytotoxicity of the effector-target interaction. Further analysis of cyclic nucleotides, in addition to a chelating agent (EDTA), demonstrated that cyclic AMP sharply diminished the cytotoxic reaction, but did not effect the binding, whereas the EDTA greatly diminished both the binding and the cytotoxicity. These analysis have enabled us to conclude that a protein receptor is involved in the effector-target interaction as is determined by the papain and trypsin sensitivity of the binding. In addition, most agents which effect cytotoxicity appear to effect a post-binding stage of cytotoxicity with only EDTA and PMA effecting a binding step. EDTA has been shown in the T cell system to abrogate binding due to its ability to bind calcium magnesium which is involved in early binding stages. The mechanism of the effects of PMA requires further elucidation.

B. The Specificity of NK Cells

The nature of the recognition by NK cells of a wide range of tumor cells and some normal cells. We previously demonstrated, by cold target inhibition and by depletion on selective tumor cell monolayers, the ability to remove reactivity against certain targets with complete maintenance of reactivity against other target cells. This has suggested that NK cells have a variety of recognition structures and that their specificity is clonally distributed. We have begun a detailed biochemical analysis of initial binding steps of purified LGL to NK-susceptible targets. We have found that solubilized material from K562 membrane inserted into lipid model membranes can efficiently inhibit effector-target cell interactions, but does not inhibit the subsequent cytolytic reaction. This model system should now enable us, to biochemically characterize the nature of the antigen(s) on the target cells and to determine the possible diversity of structures on different target cells.

C. Factors and Agents Affecting the Level of Cytotoxic Activity

Interferon has been shown to have a variety of effects on immune reactivity, including the ability to rapidly augment cell-mediated cytotoxic responses such as the reactivity of NK cells, and macrophages, or monocytes. Some of these effector mechanisms may have in vivo importance in the resistance against tumor growth or against infections by various microbial agents. To obtain better insight into the nature of the diversity of such effects by interferon, various preparations of human leukocyte interferons (12 purified or partially purified species of leukocyte α interferon) were tested for ability to augment the reactivity of NK cells and monocytes. These interferon species were tested at several antiviral titers, to determine possible quantitative differences in their ability to modulate immunological function. At higher doses of interferon (i.e. > 500 units) all of the interferon species demonstrated significant augmentation of both NK activity and of monocyte-mediated cytolysis and cytostasis. However, if low levels of interferon were imployed (10-50 units) appreciable differences among the various pure species of human leukocyte interferon were seen. Generally, a similar pattern of relative potency was seen for the cytototytic activity by both monocytes and NK cells. However, comparison of the cytolysis and cytostatic

assays demonstrated some very interesting differences. For example, interferon leukocyte γ1, β2 and β3 species strongly induced augmentation of NK and monocyte-mediated cytolysis, whereas these preparations had low potency for augmentation of cytostatic activity. Conversely, leukocyte β3 interferon generally a weak augmentor in the cytolytic assays but a high level augmentor in the cytostatic assay. Collectively these results demonstrated substantial quantitative differences in the ability of the various species of human leukocyte interferon to significantly augment levels of cell-mediated functions. Such results should have a significant impact in choosing the interferon species for appropriate clinical trials.

In addition to natural leukocyte interferons, we have examined the newly available bacterial-produced homogeneous leukocyte interferon for its augmentation of cytotoxic activity of NK cells and monocytes. As with the purified species of leukocyte interferon, its relative potency was tested at various antiviral units. In parallel experiments, recombinant interferon and natural interferon were found to have similar activities, with 10-100 units of interferon producing similar levels of augmentation. In our experiments the species specificity of recombinant interferon was maintained with no augmentation of mouse NK or monocyte functions. This demonstration that homogeneous recombinant interferon has potent effects on effector mechanisms provides a further basis for optimism regarding its potential usefulness in clinical trials.

D. Cytotoxicity by Cultured Cells

A major limitation for the detailed analysis and characterization of NK cells is that they represent a small proportion of the lymphoid cells in the peripheral organs. A potential solution to this problem was suggested by the observation that peripheral blood lymphocytes can be cultured in the presence of partially purified T cell growth factor (interleukin 2 or IL-2) and these continuously growing cell lines have two distinct cytotoxic capabilities: 1) an NK-like and 2) a polyclonally activated T-like activity. These cultures have been shown to be cytotoxic against a variety of NK-sensitive targets and against alloblasts. We have combined the ability to isolate and purify highly enriched populations of LGL and T cells with the ability to propagate these cells in the presence of T cell growth factor, and have analyzed the cytotoxic and phenotypic characteristics of the resulting cultures. Highly purified and enriched populations of LGL, which were essentially devoid of mature typical T lymphocytes as detectable by morphology or surface antigen analysis with monoclonal antibodies, were examined for 1) their growth capacity, 2) their cytotoxic capabilities against a variety of target cells, and 3) their temporal expression of surface antigens and markers in cultures. The highly enriched LGL cultures, as well as the T cell cultures, maintained rapid growth in the presence of IL-2. The T cells maintained relative stability in culture, demonstrating an increase in the ability to form rosettes at 29°C with sheep erythrocytes, and showing a slight increase in the expression of the OKT3 antigen. However, they did not express new antigens as determined by a battery of monoclonal reagents, with the exception of the expression of Ia, which has been previously reported on activated T lymphocytes. In contrast, the LGL cultures demonstrated some very significant changes in phenotype. OKT10, OKM1, and Fc receptors, are present on a high proportion of fresh LGL, whereas cultured LGL demonstrated a dramatic loss of these phenotypic characteristics which were much dimished or absent by 10 days. Conversely, the appearance of Ia antigens and

OKT3 antigens as well as the ability of a subset population of LGL cultures to form rosettes with sheep erythrocytes at 29°C became quite apparent at about the same time in culture (approximately 10 days). Analysis of the cytotoxic capacity of these cultured LGL and T cells was performed. The cultured LGL demonstrated typical killing against NK-susceptible targets, which was exhibited by both the fresh and the cultured LGL to a similar degree and their ability was boosted by interferon (a characteristic of NK activity). Both fresh and cultured NK cells exhibited typical and previously reported lectin-induced cytotoxicity. However, the fresh and cultured T cells only demonstrated lectin-induced cytotoxicity and did not demonstrate NK-like killing against the panel of NK targets. Polyclonal activation against alloblasts as well as activation and killing against anchorage dependent target cells appeared in the cultured T fractions. These cultures are of practical importance in the facilitation of the biochemical analysis of NK cells and T cells and in the determination of the controversy of several issues concerning these effector cells. It seems particularly likely that clones of NK cells and T cells will be very useful in their detailed characterization. However, the lack of an NK specific marker which is maintained in culture, makes the analysis of NK cells and T cells from a mixed population difficult and therefore requires the subpopulation analysis for proper identification of the cytotoxic effector mechanisms studied.

Significance of Biomedical Research and the Program of the Institute

Natural cell-mediated immunity may play an important role in immune surveillance against tumors. Understanding and determinating the *in vitro* role of natural immunity in human tumor systems should be very useful in the understanding of the significance of human natural cell-mediated immunity *in vivo*. The recent findings of the morphologic counterpart of NK cells greatly facilitates the studies of the mechanisms and relevance of NK cells *in vivo* and *in vitro*. The further characterization of the NK cell phenotype offers the ability to both monitor histochemically and phenotypically NK cells in clinical situations. In addition, the finding that interferon, both natural and recombinant leukocyte interferons, is a potent modulator of NK cell activity provides an important possible mechanism for therapeutic effects of interferon in clinical protocols. In addition, the finding that NK cells may be cultured in the presence of growth factors *in vitro* offers the possibility for therapeutic trials with highly purified populations of NK cells or T cells.

Proposed Course of Project

Extensive studies on natural cell-mediated immunity against tumors will be continued. Much of our efforts will be focused around the recent finding that human NK cells are LGL: 1) A more extensive phenotyping of human LGL, especially attempts to find monoclonal antibodies specific for LGL. Particular focus will be on reagents which provide insight into the lineage of these cells, especially their possible relationship to either T cells or monocytes. 2) Further characterization of the function of LGL and comparison with non-LGL which possess Fcγ receptors. We are particularly interested in determining whether LGL have functions which have been associated with mature T cells and whether these cells produce interferon or other lymphokines in response to stimuli including tumor cells, tumor antigens and polynucleotides (such as poly I:C). 3) Studies of the possible differentiation of LGL both *in vivo* and *in vitro* and whether they can

develop into a typical T cell or other lymphoid cell population. 4) Attempts at in vitro cloning of NK cells from cultures of highly purified populations of LGL. 5) A detailed examination of the biochemical mechanism involved in augmentation of NK and ADDC by interferon. Some of the biochemical mechanisms of interferon induction of antiviral resistance have been determined. We plan to apply the same approaches to study the biochemical mechanisms involved in interferon-induced augmentation of NK and other cell-mediated cytotoxic activities. 6) Further studies in nonhuman primates to further examine the in vivo behavior of NK cells. Because of the ability to isolate on Percoll gradients nonhuman primate LGL and T cell populations, in vivo studies are planned on a limited basis to examine recirculation of lymphocyte subpopulations as well as the possible in vivo differentiation of isotopically or fluorescein-labeled cells and their development into other lymphoid cell types.

Another area of continued interest in our studies of NK cells is the characterization of the specificity of their interaction with target cells. The reactivity patterns of clones of cultured NK cells should be very helpful in this regard. In addition, experiments are being performed to fractionate NK target cell membranes and characterize the nature of the structures involved in the conjugate formation between highly purified NK cells and target cells. Further separation and biochemical identification of soluble materials will be performed.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08517-06 LID

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cellular and Tumor Immunology in Experimental Animal Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Modulation of natural killer (NK) activity and macrophage functions have been examined. Macrophages can be activated in vitro to become suppressive and cytotoxic against tumor cells by lymphokines plus endotoxin. Other agents, such as interferon, poly I:C and exdotoxin alone, induce macrophages to become cytotoxic but not suppressive. During the early stages of macrophage activation there is an inhibition of both RNA and protein synthesis. Monoclonal antibodies against macrophage specific surface antigens have been developed. The pattern of tumor cell lysis by macrophages is similar, regardless of the source of effectors or the activating agent. However, the pattern of inhibition by various sugars differed depending on the target utilized. In addition, the pattern of inhibition of NK activity with sugars was not the same as with macrophage cytotoxicity. Macrophage circulation studies demonstrated that macrophages injected intravenously first go to the lung and then to the liver and spleen. NK activity can be suppressed in vitro by normal, nonactivated macrophages while macrophages activated by Corynebacterium parvum do not suppress. Congenic strains with high and low NK activity are being developed.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The general objectives of this project are a) to gain insight into the changes that occur during the process of macrophage activation, b) to determine the nature of the effects that macrophages have on normal lymphoid and tumor cells, c) to examine the interaction between tumor cells and macrophages, d) to identify and characterize macrophage subpopulations in mice, and e) to gain more insight into factors regulating natural killer (NK) cell activity. More specifically, the purpose of the projects performed this year were: (1) to dissect the mechanisms involved in macrophage-mediated suppression of lymphocyte function; (2) to examine the macromolecular events occurring in the process of macrophage activation; (3) to produce monoclonal antibodies to macrophage cell-surface antigens that could be used to identify macrophage subpopulations; (4) to study the specificity of macrophage-tumor cell interactions; (5) to compare different methods of macrophage activation for their ability to induce cytolytic and suppressor functions; (6) to determine the circulation pattern of adoptively transfered macrophages; (7) to investigate the genetic factors that determine low NK activity in mice; and (8) to study the mechanisms for augmenting and suppressing NK activity in mice.

Methods Employed: Lymphoid cells were obtained from the spleen, lymph nodes, peritoneal cavity, peripheral blood, thymus and tumor (of tumor bearing animals) of immune and normal mice. Normal and tumor cell lines were maintained in tissue culture. A variety of tumors induced by viral, chemical or unknown agents were maintained by transplantation in syngeneic animals. Immune and normal lymphocytes and tumor cells were stored in a viable frozen state. Inbred strains of mice were employed for several different allogeneic and tumor model systems. Immunological competence and/or cellular immune reactivity were measured in vitro by the following tests: lymphocyte stimulation, lymphocyte- and macrophage-mediated cytotoxicity in ^{51}Cr and $^{111}InOx$ release assays, production of lymphokines, in vitro generation of secondary cytotoxic responses, suppression of mitogen-induced and antigen induced immune functions, and inhibition of growth of lymphoma cells. Normal spleen cells from immune animals were fused with plasmacytoma tumor cells and the resulting hybrids cloned to select lines that produced monoclonal antibodies to macrophage cell surface antigens. Lymphoid populations were enriched or depleted of different subpopulations by: iron/magnet treatment, passage over adherence columns, or treatment with various antisera plus complement.

Major Findings:

I. Macrophage-Mediated Suppression

The ability of different populations of macrophages to affect the production of macrophage activating factor (MAF) by stimulated T-lymphocytes was investigated. We found that activated macrophages, infiltrating MSV-induced regressing tumors or recovered from the peritoneum of mice-injected with C. parvum, were able to actively suppress the production of MAF. MAF production by antigen-stimulated MSV-immune or alloimmune spleen cells and by normal spleen cells stimulated by Con A was susceptible to macrophage-dependent suppression to a similar extent. In contrast, resident macrophages or those elicited by light mineral oil or proteose-peptone did not affect MAF production. While suppressor cells added at the time

of the lymphocyte stimulation inhibited MAF production, the same macrophages added 4 hr after stimulation were ineffective. Therefore, it seems that the macrophages suppressed the early events of lymphocyte activation leading to MAF production.

II. Macromolecular Events Occuring During Macrophage Activation

We investigated the possibility that the functional and biochemical changes that occur during the activation of macrophages were accompanied by modifications in their macromolecular synthesis. Proteose-peptone elicited, adherence-purified macrophages from C57BL/6 mice were treated in vitro with poly I:C and their activation was evaluated by measuring cytolytic activity and glucose oxidation. Protein and RNA synthesis were measured by the incorporation into acid-insoluble material of ^{3}H-leucine and ^{3}H-uridine, respectively. Eight hours after poly I:C treatment, the RNA synthesis of the macrophages began to decrease, and by 24 hours it was 30-40% of the untreated controls. The decrease in ^{3}H-uridine incorporation could not be accounted for by a higher turnover of RNA in the activated cells or by increased cell death. Protein synthesis was also inhibited in poly I:C-treated macrophages but only after 20 hours. Despite the decrease in macromolecular synthesis, macrophages at 16 hours after treatment with poly I:C were found to be functionally activated, with high levels of cytotoxic activity against ^{51}Cr-labeled target cells and a 3- to 4-fold increase in the rate of glucose oxidation. Treatment of resident peritoneal macrophages with poly I:C led to the same pattern, a decrease in macromolecular synthesis and high cytolytic activity. To determine whether this alteration in macromolecular synthesis detected in vitro was a more general phenomenon associated with activation, we compared the RNA and protein synthesis of resident peritoneal macrophages (R-MØ) and macrophages activated in vivo by C. parvum (CP-MØ). CP-MØ showed a markedly reduced rate of RNA synthesis, ranging between 20-40% of the R-MØ. The protein synthesis, on the contrary, was not significantly different. We hypothesize that inhibition of macromolecular synthesis, particularly an early inhibition of RNA synthesis, is required for activation of macrophages.

III. Monoclonal Antibodies to Macrophage Cell Surface Antigens

We are interested in examining the activation and differentiation of macrophages. It appears that there are different subpopulations of macrophages, which vary in their stage of activation or differentiation. These subpopulations have been separated on the basis of their size, density or surface characteristics. Reports from several laboratories indicate that there also are antigenic differences among macrophages obtained from different anatomical sites and among macrophages at different stages of activation and/or differentiation. However, the antisera utilized to identify these differences were very difficult to prepare because very large numbers of adsorptions were required to make the antisera specific. Monoclonal antibodies would be more desirable because these adsorptions would not be necessary and the reagents would be against monospecific determinants. We initiated these experiments to develop monoclonal antibodies against cell surface antigens on macrophages in an attempt to provide reagents that could identify subpopulations of macrophages based on their antigenic characteristics.

Hybridomas were produced by the fusion of the mouse myeloma, NSI, with spleen cells from rats immunized with a macrophage cell line, P388D$_1$, or with bone marrow cultured macrophages that had been activated with poly I:C. After fusion, the hybrids were selected in HAT medium and then cloned and tested for the production of cytotoxic antibodies to various targets. Clones were selected that appeared to be producing antibodies specific for macrophages and further characterization was performed.

One clone, 32.50 has been previously obtained and partially characterized. This antibody reacted with a variety of macrophage cell lines and also with some other types of tumor lines. However, the pattern of reactivity with normal cells was restricted to macrophages, polymorphonuclear leukocytes, and some bone marrow cells. This antibody, in the presence of complement, would also lyse a substantial portion of pyran- or C. parvum-activated macrophages but did not eliminate the cytolytic activity of these cells. More recently we have produced two other monoclonal antibodies that appear to be macrophage-specific. Although the characterization of these antibodies has not been extensive, they do not react with normal T or B lymphocytes nor with non-macrophage tumor lines. However, they do exhibit high levels of reactivity against normal peritoneal macrophages, bone marrow macrophages and some macrophage cell lines. Further characterization is in progress to determine the range of reactivity of these antibodies and to determine whether they recognize subpopulations of macrophages.

IV. The Specificity of Macrophage-Tumor Cell Interactions

Macrophages have been shown to kill most tumor cells with a varying degree of efficiency. The question of whether there is any specificity to this interaction was reexamined. Macrophages from several strains of mice were activated by various agents and tested for their cytolytic activity against a panel of tumor targets. Regardless of the donor strain of the effector cells or the method of activation, the pattern of reactivity was similar. When several macrophage-like cell lines were tested in a similar manner, some gave a pattern that was similar to normal activated macrophages while others were distinctly different. This may reflect the origin of the cell lines from different subpopulations of macrophages or may be due to cytotoxicity by devergent effector mechanisms.

Several monosaccharides were tested for their ability to inhibit macrophage-mediated cytotoxicity and for comparison, their effects were also tested on NK activity. Different patterns of inhibition were noted with the activated macrophages, depending on the target cells employed. Only N-acetylgalactosamine inhibited cytotoxicity against L5178y, while mannose and N-acetylgalactosamine reduced the activity against RL♂1. When this was compared to inhibition of NK activity, it was noted that mannose gave reproducible inhibition of NK activity while glucose and galactose also inhibited cytotoxicity but to a lesser extent. The sugars were not toxic either to the effector cells or to the targets employed. Inhibition of cytotoxicity by sugars may provide a tool that can be used to investigate possible differences in the recognition and/or mechanism of lysis by macrophages and NK cells. The sugar inhibition data also provide evidence, for the first time, that there is heterogeneity of recognition or lysis by macrophages. Further studies are planned to explore this possibility.

V. In Vitro Activation of Macrophages to Induce Suppressor and Cytolytic Functions

Macrophages activated in vivo have been shown to be cytotoxic against tumor targets and suppressive for many phases of the immune response. However, it is not clear whether these two types of functions are manifestations of the same subpopulation of macrophages, at the same stage of activation or differentiation. We tried to determine what conditions would be necessary to activate macrophages in vitro to suppress lymphokine production and how these would compare with the conditions necessary to induce cytotoxic macrophages.

Previously we had found that macrophages elicited by proteose-peptone could be activated to become cytotoxic with lymphokines only if they were also exposed to endotoxin (LPS). However, such macrophages could be induced to be suppressive, as measured by inhibition of lymphokine production, by treatment with lymphokine alone. Therefore, lymphokine treatment itself is sufficient to induce macrophages to become suppressor cells, whereas an additional signal is necessary to induce cytolytic activity.

With a variety of other macrophage activating agents, there was a similar dichotomy between the induction of suppressor and cytolytic activities. Poly I:C, interferon, and LPS, used at higher concentrations, were capable of activating peritoneal macrophages elicited by proteose-peptone to become cytolytic. However, such treated macrophages could not suppress lymphokine production. This would suggest that different subpopulations of macrophages express cytolytic and suppressor activities and that the stimuli may selectively stimulate one or another function.

VI. Circulation Patterns of Adoptively Transferred Macrophages

It was of interest to investigate the circulation pattern of iv injected macrophages a) since in vivo studies on the suppression of NK activity by macrophages were going to be performed in parallel with the in vitro studies on suppression of NK activity (see below) and b) for comparison with the clearance pattern of iv inoculated tumor cells. Peritoneal exudate cells were labeled with ^{111}InOx, an isotope that has a very low spontaneous release (.5-1.0% release per hour), and inoculated iv into syngeneic and allogeneic mice. Peritoneal macrophages were located in the lung shortly after inoculation. Within three hours, 20-30% of the cells left the lung and went to the liver. At 24-48 hours after transfer, there was a low percentage of cells in the spleen (3-8%). When tumor cells were inoculated into mice, they were cleared more quickly from the lung and for the most part, they did not migrate to the spleen or liver. The circulation pattern of several macrophage cell lines was also examined and it was noted that they behaved very similarly to tumor cells rather than normal macrophages. Macrophages were different from spleen cells or thymocytes in their migration pattern, with a much higher percentage of the latter cell populations being found in the spleen (25-30%) after 24 hours.

VII. Modulation of NK Activity

The levels of NK activity in mice are affected by a number of factors. Identifying and understanding the mechanisms involved in increases or decreases

in NK activity would provide methods for selectively modulating this activity in vivo, thus enabling us to determine the relative importance of this function in surveillance against tumors.

A. Genetic factors in regulating NK activity. In mice, multiple genes control the expression of NK activity and experiments have been initiated to study the interactions among some of these genes. We have noted that SJL/J and A/J mice have one or more genes which are sufficient to reduce NK activity if transfered to mice with intermediate to high levels of NK activity. Attempts are now in progress to place this gene on other genetic backgrounds, to produce congenic mice with high and low NK activity.

B. Modulation of NK activity by murine macrophages. Several regulatory mechanisms have been identified which can alter the levels of NK activity. Soluble factors, such as interferon, can enhance NK activity while several classes of prostaglandins can inhibit this function. However, in most situations with altered NK activity, the mechanisms involved have not been defined. We have recently examined in detail one possible mechanism for suppression of NK activity. Adherent peritoneal macrophages, induced by thioglycollate or obtained from untreated mice, suppress NK activity when incubated with these effector cells overnight at 37°C. These macrophages do not suppress at the effector phase since addition of macrophages to the cytotoxicity assay had no effect on NK activity. Macrophages from normal mice of several strains, including nude mice, were shown to have suppressor activity. In contrast, macrophages that have been activated in vivo with C. parvum do not suppress NK activity. Several macrophage-like cell lines also did not suppress NK activity. Both spontaneous and interferon-boosted NK activity were inhibited by the addition of as few as 10% macrophages. Since there is low NK activity in the peritoneal cavity, this may represent one mechanism by which NK activity is naturally suppressed.

Significance to Biomedical Research and the Program of the Institute:

The immune response to neoplasia is very complex and involves not only the interaction between host effector cells and tumor cells, but also interactions among the various subpopulations of leukocytes. Some cells and/or factors may serve to amplify the immune response, while others may suppress it. We have been studying some of these interactions on several levels to learn more about the host effector mechanisms that are antagonistic to the growth of the tumor and about the factors or cells that regulate the development of these responses.

Macrophages are capable of modulating the host's response to the tumor, both by their ability to act as accessory cells in the development of an immune response and by their capacity to suppress development of immune functions. We have found that macrophages exert their suppressor activity very early during the development of the immune response and may act by inhibiting protein synthesis. A delicate balance may exist between the accessory function and suppressor function of macrophages and the ability of a host to deal with a tumor may be critically affected by the net state of balance between these regulatory fucntions. Understanding all these interactions, what influences them and how they can be modulate to the benefit of the host, will give us information that we can use to assist the host in dealing with its tumor.

Macrophages can also have direct antitumor activity. Studies on the process by which macrophages become activated have indicated that there are multiple stimuli which are required for macrophages to become cytolytic. In addition, there appears to be several different stages of differentiation which may be represented by distinct subpopulations of macrophages. Identifying the mechanism of macrophage activation and the subpopulations involved in this process should provide information that can be used to establish immunotherapy regimens.

Natural killer cells may have an important role in the protection of the host from tumor induction (immune surveillance) or metastasis, or in the antitumor response after different types of immunotherapy. Studies on the mechanisms regulating their functional activity should not only provide insight into the control of the cytotoxic activity of these cells but also provide the means to modulate the levels of activity in vivo, thereby enabeling more detailed experiments about their in vivo relevance to be performed.

Proposed Course of the Project:

During the next year we will continue to examine the processes involved in activation of macrophages and the specificity of macrophage-tumor cell interactions. In addition, we will continue to attempt to identify macrophage subpopulations, by their functional activity or by monoclonal antibodies or physical separation. Furthermore, modulation of NK will be investigated by studying the suppression of NK activity by macrophages and by examining the genetic regulation of NK.

Specificity of the macrophage-tumor cell interactions will be studied using the inhibition of cytotoxicity by sugars in conjunction with conjugate formation. We plan to develop a single cell assay which will enable us not only to visualize the binding of macrophages to tumor cells, but also to determine the fate of the tumor cell after binding. This will help to provide information of the effect of the sugars, i.e. whether they inhibit binding or whether they have an effect on the cytolytic phase. In addition, the panel of sugars that is being examined will be enlarged to include other molecular forms of the monosaccharides (such as hexose-6-phosphates). Preliminary testing has suggested that these may also have similar effects.

Work will continue to identify different subpopulations of macrophages. In addition to the monoclonal antibodies against macrophage cell-surface antigens that have already been produced, additional hybridomas will be initiated in an attempt to select antibodies against other subpopulations. In conjuntion with this work, other methods of distinguishing subpopulations will be explored, such as the physical separation of cells or the use of other antisera, such as anti-Ia, to determine whether the subpopulations that are being identified are similar. Further attempts will be made to characterize the monoclonal antibodies that have been produced. This will involve treatment of macrophages that are used either in vitro for studies on the activation process or in vivo for evaluation of functional activity.

Macrophage activation will be examined from several different aspects. Since it appears that various agents activate macrophages for some but not all functions, the mechanisms for the divergent effects will be examined. Mice with

different genetic backgrounds, some expressing macrophage defects, will be a source of macrophages to determine whether these function, i.e. suppression, cytostasis, cytolysis, and the kinetics of activation, can be discriminated. In addition, resident peritoneal macrophages and macrophages from the bone marrow will be compared, to determine whether some of these differences can be attributed to the stage of differentiation of the macrophages.

Modulation of NK activity will be investigated using several different experimental approaches. The breeding program which was initiated during the past year will be continued in an attempt to learn more about the genetic factors that regulate NK activity and about the interaction between these factors. Low NK activity in one strain of mice (SJL/J), appears to be mediated by one gene. We will attempt to place this gene onto strains of mice that have intermediate to high NK activity so that strains of mice congenic for high and low NK activity can be developed. This will provide models to investigate the in vivo role of NK activity.

Suppression of NK cells by macrophages will also be examined. The two types of suppressor cells that have been described, those which directly inhibit the effector phase and those which suppress only after they have been preincubated with the effector population for 18 hours, will be compared, to determine the similarities and differences in their characteristics and the mechanisms of their effects. Furthermore, we will perform adoptive transfer experiments to determine whether these suppressors can inhibit NK activity in the spleen and other organs of recipient mice. Studies on the ability of macrophages to inhibit the in vivo clearance of tumor cells, a function that is part mediated by NK cells, will be performed.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05557-13 LID

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cellular Immune Responses in Experimental Tumor Systems and Cancer Patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human and rat natural killer (NK) cells have been studied in detail. NK activity in these species has been shown to be mediated by large granular lymphocytes (LGL) and LGL have also been detected in a variety of other species. Rat LGL were shown to share surface markers with both T cells and macrophages and the number of these cells was appreciably increased in nude rats. Clones of cultured mouse NK cells have been produced and these also share some antigens with T cells. The mechanism of augmentation of rat NK activity by interferon (IFN) was analyzed and shown to involve increases in target binding by LGL and also increased lytic activity. In contrast to boosting of NK activity, IFN had no effect on cytotoxicity by immune mouse T cells. Depression of NK activity by various agents was shown to be due either to altered regulation or to reduction in the number of effector cells. A role for proteases and not for reactive oxygen species in the mechanism of NK activity has been demonstrated. NK cells have also been shown to produce IFN in response to a variety of stimuli. NK cells were reactive against primary mouse mammary tumors as well as against tumor cell lines and further evidence has been obtained for an in vivo role of NK cells, including their possible activity in immune surveillance.

Project Description:

Objectives: The objectives of this project are: 1) To study natural cell-mediated immunity to tumors in rodents and in man, and to determine the characteristics of the effector cells, the factors regulating the levels of activity and the role in resistance against tumor growth; 2) to evaluate patients with lung and breast cancer for the correlation between immune parameters and clinical course of disease; and 3) to investigate the effects of immunotherapeutic manipulations on the immune reactivity of cancer patients.

Major findings:

I. Natural Cell-Mediated Immunity Against Tumor Cells

A. Characterization of NK Cells

In last year's report we described a major new finding: the intimate association between human and rat NK cells and cells with a characteristic morphology termed large granular lymphocytes (LGL). This association, coupled with the ability to purify LGL by centrifugation on discontinuous density gradients of Percoll, has provided the basis for a large series of new studies on the characteristics of NK cells. Our recent progress with the characterization of human LGL is summarized primarily in report Z01 CB 08503-01. In addition, we have examined the blood or spleen cells of a variety of animal species for the presence of LGL and for the parallel expression of NK activity or antibody-dependent cell-mediated cytotoxicity (ADCC) activity. LGL and NK and/or ADCC activities have been detected in most of the species examined, including rats, nonhuman primates, dogs, cats, sheep, cattle, pigs and chickens. The mouse initially stood out as the main exception, with clear NK and ADCC activities but few if any detectable LGL in the spleen or blood. However, recently we have found that this was due to the need for alterations in the staining procedure and to the smaller size of mouse LGL. In all of the species studied, the frequency of LGL among peripheral blood mononuclear cells varied between 1 and 10%. We have selected the rat as the experimental rodent species for detailed characterization of LGL. The LGL frequency from various organs was shown to be: peripheral blood = lung > spleen > peritoneal exudate > lymph node. Few if any LGL were detected in the thymus or bone marrow. This pattern was similar to the distribution of NK acticity, except for the lungs which had no detectable cytotoxic acticity. It has been possible to enrich or deplete for rat LGL on Percoll gradients, and only enriched LGL populations bound selectively to NK-susceptible target cells and caused their lysis. In parallel with the high NK activity in the blood or spleen of nude, athymic rats, LGL frequency was shown to be increased 2-5 fold in these rats.

With the use of monoclonal antibodies and continuous flow microfluorometry, we have examined the cell surface markers on rat LGL and compared them with monocytes, typical small T cells, and granulocytes. As in the human studies, the rat LGL were found to be an antigenically distinct population of cells which share some characteristics with monocytes, T cells, and granulocytes. Essentially all of the LGL expressed the W3/13, OX-8, leukocyte-common (L-C), and asialo GM1 antigens. A portion of these cells were also positive for ART-1^a. In contrast, few LGL expressed Ia, W3/25, surface immunoglobulin, or Thy 1.1 anti

gens. Monocytes had a similar pattern but were OX-8 negative, and T cells expressed ART-1^a, W3/25 and/or OX-8.

To obtain large numbers of homogeneous populatins of NK cells, for characterization and for possible in vivo therapy experiments, an emphasis has been placed on the in vitro propagation of mouse NK cells. It has been possible to maintain the proliferation of cytotoxic cultured lymphoid cells (CLC) for 1-12 months in the presence of crude or mitogen-depleted T cell growth factor. Clones from these CLC were established by either limiting dilution or soft agar techniques. Most of the clones had strong cytotoxic activity against a variety of syngeneic and allogeneic tumor target cells. The clonal populations generally exhibited a more restricted pattern of cytotoxic activity than the parental CLC and the pattern varied among the clones. One group of clones reacted preferentially against lymphoid tumor target cells and the others reacted strongly against both lymphoid and solid tumor targets. The clones expressed some markers associated with NK cells (asialo GM1, NK2, T200) and also some characteristic T cell markers (Thy 1.2, Lyt 2). As further indications that the cytotoxic clones were related to NK cells, we found that their activity was augmented by interferon and that they also had ADCC activity.

B. Regulation of NK Activity

There is considerable evidence that the activity of NK cells is subject to a variety of regualtory factors. During the past year we have continued detailed studies on the mechanisms involved in augmentation and inhibition of NK activity.

Interferon (IFN) is the major mediator of augmentation of NK activity. We have performed studies with rat LGL, to analyze the mechanism of the effects of IFN on NK cells. These studies were done primarily with cells from nude rats, since they have high numbers of LGL which can be purified to a higher degree than is possible with cells from euthymic rats. In a single cell cytotoxicity assay, IFN pretreatment of LGL was shown to have several effects, increasing: 1) the percentage of LGL which form conjugates with target cells, 2) the percentage of conjugate-forming cells which kill, and 3) the kinetics of lysis. The effects of IFN on one or another of these parameters varied with the target cell tested.

In order to further evaluate the in vivo role of NK cell activity in various situations and to possibly achieve better therapeutic effects with IFN or IFN inducers, it is important to develop protocols for optimal and sustained augmentation of NK activity. One of the main limitations in previous studies was the relatively low and transient levels of circulating IFN levels that could be induced. We have recently perfomed studies in mice with blue tongue virus (BTV), which has been reported to induce very high serum levels of IFN. We have confirmed this and have detected IFN for at least 7 days after inoculation. The NK activity at 1-3 days after administration was boosted only to a degree comparable to that induced by poly I:C or lymphocytic choriomenengitis virus, despite the considerably lower levels of IFN induced by these agents. However, the augmentation of NK activity by BTV persisted for more than 7 days, substantially longer than that seen with one dose of other agents. In an attempt to achieve even more prolonged boosting of NK activity, we have repeatedly inoculated mice with poly I:C at various intervals. Some mice, in addition, were given prostaglandin $F_{2\alpha}$, which doesn't interfere with NK activity and has been reported to reduce or

eliminate the problem of hyporesponsiveness to IFN inducers after repeated exposures. A protocol of twice weekly administration of poly I:C plus prostaglandin appears promising for sustained augmentation of NK activity, with significantly elevated levels still detectable after 3 weeks. Studies are now in progress to determine the length of time such augmentation can be maintained.

It has been of considerable interest to determine whether the ability of IFN to augment cytotoxic reactivity is limited to only certain types of effector cells. It was reported almost ten years ago that IFN pretreatment of mouse alloimmune spleen cells would augment their specific activity. However, those studies were performed prior to knowledge of NK cells and utilized tumor target cells. Our concern as to whether IFN could augment cytotoxic reacticity of immune T cells was accentuated by consistently negative results of IFN pretreatment of in vitro generated human cytotoxic alloimmune T cells. We have therefore repeated the studies with mouse cells, after in vivo or in vitro alloimmunization. When NK-resistant target cells were used to assess cytotoxic T cell activity, IFN pretreatment of the effector cells had no detectable effect. It therefore seems likely that IFN can not augment specific T cell-mediated cytotoxicity and that any effects of IFN on a population containing immune effector cells are probably attributable to the augmented reactivity of NK cells.

We have also continued our studies to determine the mechanims involved in spontaneous or treatment-induced depression of NK activity. Depression in NK activity has been induced by: a) estradiol or diethylstilbestrol, b) X-irradiation, c) cyclophosphamide, d) pyran copolymer, e) Corynebacterium parvum, and f) intravenous inoculation of antibodies against asialo GM1.

It was initially thought that depression of NK activity by estrogenic hormones was due to osteosclerosis and a reduction in the number of stem cells for NK activity. However, we have found that levels of NK activity vary with the levels of circulating estradiol and it seems more likely that the depression of NK activity is due to a direct or indirect pharmacologic effect on NK cells. We have used two different approaches to determine whether these hormones have direct effects on NK cells. First, in studies with human mononuclear cell subpopulations separated on Percoll gradients, we have found that LGL and T cells have no detectable receptors for estradiol, whereas monocytes have considerable binding activity. In addition, in studies with rats treated with diethylstilbestrol, we have observed depressed NK activity, particularly in the spleen but also in the blood, but little effect on the precentage of LGL. Thus, our present evidence suggests that these hormones do not directly act on NK cells but act indirectly to alter the regulation of their activity. We are currently evaluating the hypothesis that the depressed NK activity is mediated by the induction of macrophage suppressor cells.

NK activity in mice and rats is strongly depressed by treatment with X-irradiation or cyclophosphamide. However, we have observed that treatment of such animals with poly I:C or exposure of their spleen cells to IFN results in levels of NK activity similar to that of normal mice. The persistence of NK cells after the depressive treatments has been confirmed by enumeration of LGL in rats. Thus, with these treatments also, the depression appears to be due to altered regulation of NK activity rather than to a reduction in the size of the NK cell population.

A different pattern of results has been seen in rats treated with anti-asialo GM1. It has been reported that such treatment of mice results in a strong depression of NK activity. We have confirmed this in rats as well as mice. The reduced NK activity has been paralleled by a large decrease in the percentage of LGL in the blood and spleen, suggesting that this treatment has a direct effect on the NK population.

Another type of negative regulation was found in studies of short-term incubation of human peripheral blood mononuclear cells. After incubation for 1-2 hours in medium lacking human serum, increased NK activity was seen. This seemed to be due to release from inhibition by human serum factors, since incubation in autologous or allogeneic serum prevented the augmentation and at high concentrations produced inhibition of activity. The serum-mediated effect appeared attributable to the degree of binding of labile IgG to the cells and could be reduced by selective depletion of IgG from the serum. Human or rabbit monomeric IgG was found to efficiently inhibit the culture-induced augmentation of NK activity and the inhibitory IgG had properties consistent with those previously described for cytophilic IgG. The overall pattern of results suggested that this mechanism may be involved in negative regulation of NK activity in vivo.

C. Mechanism of Cytotoxicity by NK Cells

The mechanism of cytotoxicity by NK cells has not been clearly elucidated. Several possibilities have been considered, including a role for phospholipase A_2, proteases, and reactive oxygen species. Some evidence for participation of phospholipase A_2 has been obtained by others in this laboratory (see Z01 CB 08501-02). Other investigators have suggested an important role for serine proteases, since various protease inhibitors interfered with cytotoxic activity. We have therefore examined this possibility in detail. Highly purified populations of human LGL, with no detectable contamination by monocytes, could be shown to produce a fibrinolytic enzyme with characteristics of plasminogen activator. Furthermore, addition of a wide range of selective protease inhibitors to the cytotoxicity assay resulted in strong inhibition of NK activity by some inhibitors. The pattern of inhibition was highly suggestive of an important role for a protease(s) with the characteristics of chymotrypsin. It will be important to determine whether both protease and phospholipase A_2 independently contribute to lysis by NK cells or, more likely, whether these enzymes have important, sequential effects.

The possibility of a role for reactive oxygen species in NK activity was examined by analysis of the ability of human LGL to undergo an oxidative burst in response to a variety of stimuli. Entirely ngative results were obtained with LGL, whereas monocytes had expectedly high levels of production. Thus this potential mechanism for cytotoxicity does not seem to be involved in NK activity.

D. Production of Interferon by NK Cells

There have been some suggestions that NK cells can react against tumor cells not only by cytotoxicity but also by production of IFN. This possibility, of IFN production by NK cells, has been studied in detail by incubating highly enriched populations of human or rat LGL in vitro with tumor cells or other stimuli and measuring the levels of IFN in the culture supernatants. After

overnight incubation, LGL, but not typical T cells, produced appreciable levels of IFN in response to NK-susceptible tumor cell lines and also to viruses (influenza and herpes simplex), poly I:C, BCG, Corynebacterium parvum, and to mitogens (PHA, Con A, Staph enterotoxin). T cells, in the presence of accessory monocytes, did produce detectable levels of IFN in response to mitogens, but only after longer periods of incubation. It was of interest that the IFN produced by human LGL was heterogeneous, varying with the type of stimulus. By typing with antisera to alpha (leukocyte), beta (fibroblast) and gamma (immune) IFNs, neutralization was seen mainly with anti-alpha IFN. However, the majority of the IFN induced by mitogens and, with some donors, the IFN induced by influenza virus, was typed as gamma. Con A also appeared to induce the production of some beta IFN. Thus NK cells appear to have the capacity for self-regulation, being able to respond to certain stimuli by producing IFNs that in turn can agument their reactivity. These observations also indicate that NK cells may have a broader range of biologic effects than were initially appreciated, with a potential for directly producing an antiviral protein and for affecting the activity of other immune effector activities that are responsive to IFN.

E. Reactivity of NK Cells Against Primary Tumors

In contrast to the many studies of NK activity against a wide variety of tumor cell lines, there is very little information about the susceptibility of primary tumors to lysis by NK cells. An earlier study from this laboratory with primary AKR lymphomas provided the first indication that some primary mouse tumors had detectable susceptibility to NK activity. To examine this important issue further, we have performed studies with spontaneous mammary tumors of C3H mice. The majority of the tumors tested had significant, although usually quite low, susceptibility to lysis by normal syngeneic as well as allogeneic spleen cells, particularly in an extended, 18 hr, ^{51}Cr release cytotoxicity assay. The levels of lysis were significantly augmented by pretreatment of the effector cells with IFN. These observations indicate the potential for a role of NK cells in resistance to growth of such primary tumors. Studies are in progress to determine the basis for the degree of susceptibility of the primary tumor cells to natural cell-mediated cytotoxicity and particularly to identify procedures that could augment their sensitivity to lysis. Environmental factors have been found to play an important role, with susceptibility increasing after culture for at least 2 weeks in vitro and decreasing to undetectable levels after transplantation into normal syngeneic mice.

F. In Vivo Relevance of NK cells

During the past year, a major emphasis has been placed on the further evaluation of the possible role of NK cells in in vivo resistance to tumor growth. In addition to further studies on the in vivo reactivity of NK cells against transplantable tumor cell lines, we have initiated studies with primary tumors and on carcinogenesis by urethane or X-irradiation.

1. In vivo activity against tumor cell lines

We have continued to assess in vivo NK activity by measurement of the rapid elimination from the lungs of intravenously inoculated radiolabeled tumor cell lines. We previously showed that intravenous transfer of normal spleen

cells or bone marrow cells could restore, at least in part, the depressed in vivo resistance induced by cyclophosphamide. We have now shown that the spleen cells required for this in vivo transfer of resistance have the characteristics of NK cells, being nonadherent and bearing the NK cell-associated antigen, asialo GMI. Using lethal X-irradiation as another procedure to depress in vivo NK reactivity in rats as well as in mice, we have found that transfer of spleen cells was also able to restore resistance.

These protocols have provided a good system for analyzing for the first time the possible in vivo effects of human NK cells. After depression of NK activity in nude mice, pruified populations of human LGL, which have been shown to be highly enriched for NK activity,. were transferred and the mice were then challenged with radiolabeled human or mouse tumor cells. Such transfer of human LGL was able to increase the clearance from the lungs of the human NK-sensitive tumor cells, but had no effect on clearance of mouse tumor cells that are resistant to human NK activity.

Similar studies have been performed with transfer of mouse and human cultured lymphoid cells (CLC) that have NK-like cytotoxic reactivity. Such mouse or human CLC, when admixed with NK-sensitive tumor target cells and inoculated subcutaneously, reduced the survival of the tumor cells, as measured by local retention of radiolabel in our isotopic footpad assay, or by the incidence and rate of growth of detectable tumors. Systemic transfer of CLC had less impressive anti-tumor effects, perhaps because of problems with limited circulation or survival of the transferred cells, but some significant effects were observed. The results indicate some potential to the approach to immunotherapy by production and transfer of large numbers of cytotoxic CLC.

The in vivo radioisotopic assay has also allowed assessment of the possible in vivo significance of in vitro detected suppressor cells for NK activity. This has been studied by two types of protocols: a) In the protocol of transfer of normal spleen cells to mice with treatment-induced depression of resistance to challenge with radiolabeled tumor cells, intravenous administration of adherent suppressor cells from low NK-reactive mice substantially interfered with reconstitution. b) Intravenous administration of adherent peritoneal cells with suppressor activity to normal, untreated mice, when injected simultaneously with, or a short time after, radiolabeled tumor cells, interfered with their clearance. Furthermore, in this latter protocol, the transfer of cells with suppressor activity could be shown to result in a higher number of detectable pulmonary metastases.

2. In vivo reacticity against primary tumor cells

To begin to obtain direct evidence for a possible role of NK cells in in vivo resistance to primary tumors, we have recently initiated experiments with radiolabeled primary C3H mammary tumor cells. As with NK-susceptible tumor cell lines, inoculation of such primary cells into mice which varied in their levels of NK activity resulted in a parallel difference in the degree of clearance from the lungs of syngeneic mice. Thus, although the primary mammary tumor cells have only low levels of susceptibility to lysis of NK cells in vitro, this appears sufficient to play a role in vivo.

3. Possible role of NK cells in immune surveillance

As one model of primary carcinogenesis in mice, we have injected urethane into young mice of various strains. Only some strains, particularly A/J mice, developed lung tumors after urethane treatment. In these susceptible mice, urethane also was found to cause an early, profound depression in NK activity. In contrast, urethane treatment of strains of mice that were resistant to pulmonary carcinogenesis did not cause detectable depression of NK activity. Administration during the latent period of normal bone marrow cells, according to a protocol known to reconstitute NK activity, could interfere with the subsequent development of lung tumors in A strain mice. Thus, it seems likely that depression of NK activity is one of the necessary effects of urethane in order to achieve detectable pulmonary tumorigenesis.

As a second model system, C57BL/6 mice were treated with a schedule of multiple, low doses of X-irradiation, which is known to be highly effective in inducing thymic lymphomas in this strain. Such treatment was found to result in a substantial deficit in NK activity. This depressed NK activity could be restored by transfer of normal bone marrow cells, a procedure which has been shown to interfere with radiation-induced leukemogenesis. In contrast, transfer of bone marrow from beige mice did not restore NK activity. The leukemogenic effects of X-irradiation in beige mice were compared with those in heterozygous littermates, which have normal NK activity. The rate of tumor development thus far has been appreciably more rapid in the NK-deficient beige mice. Thus, NK cells do appear to have some role in the surveillance against radiation-induced lymphomas.

II. Monitoring of NK Activity and Other Immunologic Parameters in Cancer Patients

In collaboration with investigators at the Uniformed Services University for Health Sciences and Naval Medical Research Institute, a laboratory has been set up for detailed immunologic monitoring of cancer patients. Procedures have been established to standardize and control the assays and to minimize various sources of technical variations in results. A major emphasis has been placed on the use of cryopreserved cells: a) As standard controls in all experiments: peripheral blood mononuclear cells from several normal donors, obtained by leukopheresis, have been cryopreserved and aliquots from three donors are tested in each assay, in parallel with test specimens. By direct comparison of test results with these standards, this allows correction for day-to-day variations in levels of reactivity in each assay. b) As frozen target cells: in the cytotoxicity assays, the target cells were found to fluctuate in their susceptibility to lysis or growth inhibition. Cryopreservation of large batches of target cells has allowed the use of aliquots as targets in each experiment and has thereby considerably reduced the source of variability. c) Cryopreservation of effector cells: For all immunologic assays except that for NK activity, it has been possible to cryopreserve peripheral blood mononuclear cells from all patient specimens and test them later. This will permit the simultaneous testing of a whole series of repeated specimens from a given patient, obtained before and at various times after an experimental treatment, thereby eliminating variation due to day-to-day changes in assays. In addition, procedures have been set up for rapid computer entry of laboratory and clinical data and for display and analysis of the test results.

The following series of assays have been established for monitoring of immunologic reactivity of cancer patients: a) NK activity; b) monocyte-mediated inhibition of growth of tumor target cells; c) lymphoproliferative (LP) responses to mitogens and in mixed leukocyte cultures; and d) enumeration of lymphoid cell subpopulations, according to morphologic and cell surface markers: this includes the determination of percentage of LGL, of cells forming rosettes with sheep erythrocytes, and of cells reacting with various monoclonal antibodies (OKT3, OKT4, OKT8, OKT10, OKM1, anti-Ia) as measured with the fluorescein-activated cell sorter.

To be able to consistently have sufficient lymphoid cells from cancer patients for the measurement of multiple immunologic parameters, and to obtain dose response and other detailed information, procedures have been developed for miniaturization of some of the assays. It has been possible to adopt the procedures for performance of LP assays and of growth inhibition assays to mini-wells, which utilize only one-tenth the volume of medium and number of cells as those used in more conventional micro-assays. The incubation of the cells in the mini-wells is performed with the test plates inverted, with all of the cellular interactions thereby taking place in hanging droplets. The results obtained in such assays have been highly correlated with those obtained in parallel tests performed under conventional conditions, and therefore the patient specimens are now being routinely tested by the new procedure.

These assays are being utilized to monitor patients in the following clinical protocols:

A. Prognostic Value of Assays of LP Responses

In previous studies, we have found that measurement of LP responses in mixed leukocyte cultures (MLC), by postoperative patients with carcinoma of the lung or breast, appeared to have prognostic significance. In particular, depressed MLC reacticity in patients with stage I lung cancer was associated with a significantly shorter disease-free interval than that of patients with MLC reacticity in the the normal range. In most patients with depressed reactivity in MLC or with depressed LP responses to the mitogens PHA or Con A, it was possible to show that the presence of either adherent or nonadherent suppressor cells accounted, at least in part, for the abnormal functions. We have therefore recently set up procedures to analyze suppressor activity in more detail, test LP reacticity in the presence of inhibitors of either prostaglandin synthesis or of histamine (both of which have been incriminated in LP supppresion), and to determine whether these parameters correlate with the subsequent clinical course of the cancer patients.

B. Effects of BCG on Immunologic Reactivity of Cancer Patients

Although many clinical immunotherapy trials have been perfomed with BCG, few have been clearly successful. One of the main limitations has been that treatment protocols were empirical, since there is little information available on the effector mechanisms that are relevant to the possible therapeutic effects. Because of the possible rolls of NK cells and of cytotoxic monocytes in resistance against tumor growth, we have been monitoring these as well as other immunologic activities in patients before and after receiving one inoculation of BCG. Suprisingly, in view of the known ability of BCG to augment NK cell and monocyte-

mediated cytotoxicity in mice, in our initial clinical study no augmentation, but rather depression, of reactivity was observed. Consistent with such negative results, no increase in levels of serum IFN was seen. This raised the possibility that the Pasteur Institute BCG preparation being used was deficient in these biologic activities. In support of this possibility, other strains of BCG were found to induce systemic augmentation of NK activity in mice, whereas intraperitoneal inoculation of Pasteur BCG produced only local augmentation. A further clinical study was therefore set up to compare the immunologic effects of the two other BCG preparations, Tice and Connaught. The testing of specimens from this protocol has just been completed and data analysis remains to be done.

C. Effects of Purified Recombinant Interferon on Immunologic Reactivity of Cancer Patients

Recently much attention has been focused on the possible therapeutic efficacy of IFN. However, there is little information as to whether such effects may be mediated by direct anti-tumor effects of IFN or by the ability of this agent to augment host resistance. In view of the clear demonstration that IFN can substantially boost NK activity, one major hypothesis is that this is the mechanism for the clinical benefits from IFN. Alternatively, the effects of IFN on monocyte-mediated cytotoxic activity or other immunologic functions might be important. It therefore is necessary to determine the optimal doses and schedules of IFN administration for alteration of each of these parameters. Such an effort has recently begun with the highly purified recombinant leukocyte IFN A preparation of Hoffmann-La Roche. Patients with advanced cancer are being given a fixed dose of IFN, ranging from 1 million units to over 100 million units, in two divided daily injections or three times per week, for 28 days. These patients are being carefully monitored for the effects of the treatment in the range of immunologic parameters listed above. Preliminary analysis of the data regarding NK activity and serum IFN levels indicates no augmentation in either parameter. It will be important to determine the reason for these unexpectedly negative results, since this may have major implications for the clinical efficacy of this preparation.

Significance to Biomedical Research and the Program of the Institute:

Natural cell-mediated immunity may play an important role in immune surveillance against tumors. Understanding of this phenomenon and determination of its *in vivo* role in mice and rats should be very useful for the understanding of the significance of human natural cell-mediated immunity. The recent findings of a morphologic counterpart to the functional activity of human and rat NK cells should greatly facilitate studies on the frequency of these cells under various situations, their *in vivo* circulation patterns, their differentiation and relationship to other cell types. Findings that IFN plays a central role in augmentation of the activity of NK cells, provides an important new mechanism for potential therapeutic effects by this agent and development of protocols for optimal and persistent augmentation of NK activity may result in more effective therapy of cancer patients.

Proposed Course of Project:

Extensive studies on natural cell-mediated immunity against tumors will be continued. Most of our efforts will be directed towards a more detailed understanding of factors regulating the activity of NK cells and towards further investigation of the in vivo role of NK cells in reactivity against primary tumors and in immune surveillance.

In our further studies on regulation, we will try to determine optimal protocols for selective and sustained augmentation or depression of NK activity. These protocols should be very useful for our studies on the role of NK cells in the development of primary tumors. We will also examine in detail the mechanisms involved in depression of NK activity by various treatments, since these may lead to new strategies for restoring NK activity. Experiments will also be performed in rats, to determine whether purified populations of LGL can transfer NK activity and if so, the factors influencing the efficacy of transfer. Such experiments may also be quite useful to provide further insight into the cell lineage of NK cells, since we will examine whether differentiation into T cells or other cell types can occur.

To evaluate the role of NK cells in host resistance against tumors, the following types of studies will be performed: a) Further studies with primary mouse mammary tumors. Efforts to further characterize the effector cells and to determine the factors involved in susceptiblility or resistance of these cells to lysis by these effector cells. A particular focus will be whether certain treatments could be devised to augment susceptibility of primary tumor cells to lysis. This possible in vivo reactivity of NK cells against such primary mammary tumor cells will be initially assessed by the clearance of radiolabeled tumor cells in syngeneic normal and tumor-bearing mice. b) Experiments on the role of NK cells in primary carcinogenesis by urethane will be continued. The main objective will be to determine more clearly whether the development of lung tumors in mice is dependent on depressed NK activity and whether selective restoration of NK activity will interfere with carcinogenesis. c) Similarly, experiments with radio-induced lymphomas in normal and beige C57BL/6 mice will be continued to more clearly ascertain the role of NK cells in resistance. d) A series of experiments are being initiated in rats, to examine the possible role of NK cells in resistance against the induction of primary mammary tumors by methylnitrosourea (NMU). Experiments will include the selective depression of NK activity by repeated inoculations with anti-asialo GM1 and attempts to selectively restore NK activity in carcinogen-treated rats by administration of LGL.

Immunologic monitoring of cancer patients will be continued. The main protocol will continue to be the evaluation of the effects of IFN preparations and IFN-inducers on the levels of NK activity and on other immunologic parameters. We will also continue to study the possible role of suppressor cells in immunodeficiency in cancer patients and the possible prognostic significance of these observations.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08501-02 LID

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Roles of Phospholipid Metabolism and Monocyte Function in Tumor Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Hoffman	Senior Investigator	LID	NCI
Others:	J.Y. Douillard	Visiting Fellow	LID	NCI
	P.P. Bougnoux	Visiting Fellow	LID	NCI
	Z.L. Chang	Visiting Fellow	LID	NCI
	B. Schwartz	COSTEP	LID	NCI
	D. Halpert	COSTEP		

COOPERATING UNITS (if any)
FDA, Biological Structures Branch; NIMH, Laboratory of Clinical Science; National Naval Medical Center Tissue Bank; USUHS, Tumor Immunology Unit

LAB/BRANCH
Laboratory of Immunodiagnosis

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Phospholipid metabolism was found to play a significant role in natural killer (NK) activity. Transmethylation and phospholipase A_2 action were demonstrated to represent important changes in phosphatidylcholine metabolism observed during effector:target interaction and cell lysis. These changes in phospholipid metabolism, and others including effects on the CDP-choline pathway, induced by interferon and the tumor promotor phorbol myristate acetate (PMA), were studied in detail.

Growth inhibitory activity (GIA) was studied in normal individuals and cancer patients. Interferon and PMA were found to exert effects in this system. A novel assay for GIA based on inhibition of alkaline phosphatase activity was developed.

Efforts have been made to develop hybridomas producing monoclonal antibodies directed against cell surface components of human monocytes and natural killer cells.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To study the role of lipid metabolism in the cell membrane in regulation of the immune functions of human mononuclear cells (lymphocytes and monocytes). To examine the role of these cells in spontaneous cytotoxicity or cytostasis of tumor cells.

Major Findings:

I. Phospholipid metabolism.

A. Natural killer activity. Initial studies from this laboratory established a role for phospholipid metabolism in natural killing. Transmethylation and phospholipase A_2 activity were both found to increase during NK cell-target cell interaction. This evidence was obtained in large part through the use of agents which inhibited transmethylation enzymes (3-deazaadenosine, 3-DZA) or phospholipase A_2 (chloroquine, quinacrine, corticosteroids, and Rosenthal's inhibitor). Measurements of the release of radiolabeled arachidonic acid from the cell and analysis of the total phospholipids remaining within, confirmed the involvemnt of these pathways by quantifying the end-products of these reactions. Using more refined techniques of thin-layer chromatography (TLC) with multiple solvents, data were obtained to show that phosphatidylcholine, mainly generated via the transmethylation pathway, served as the substrate for phospholipase A_2. Similar studies using purified mononononuclear cell fractions enriched for large granular lymphocytes (LGL), known to be responsible for NK actiivty, showed that these cells demonstrated the same properties of transmethylation and phospholipase A_2 activation.

B. Interferon. Since interferon has been shown to have wide-reaching effects on various immune functions, including boosting of NK activity, its effect on phospholipid metabolism was examined. Interferon was found to increase endogenous phospholipase A_2 activity, as measured by release of prelabeled arachidonic acid, in peripheral blood mononuclear cells, monocytes, purified T cells and LGL. Paradoxically, this effect was least prominent in LGL.

In contrast to the effect observed during NK-target cell interaction, IF did not change the total amount of arachidonic-acid containing lipids. Fractionation of labeled lipids by thin-layer chromatography of peripheral blood mononuclear cells showed a redistribution of unsaturated fatty acids, with decrease of labeled phosphatidylcholine concomitant with an increase in labeled neutral lipids. In experiments in which the effect of interferon on the transmethylation pathway was examined, decreased incorporation of tritiated methionine into phosphatidylcholine was observed. This provided support for the hypothesis that interferon increased phospholipase A_2 activity principally on substrate derived from the transmethylation pathway.

In related experiments, the tumor promotor phorbol myristate acetate (PMA) was observed to increase phosphatidylcholine synthesis via the CDP-choline pathway. Interferon was found to abrogate this increase.

II. Functions of monocytes in tumor immunity.

Studies of inhibition of tumor growth by peripheral blood monocytes were continued in a variety of assay systems. Growth inhibitory activity (GIA), measured by effects on tritiated thymidine incorporation, was found to be increased after pretreatment of effector cells with PMA or IF (at certain effector/target ratios, at specific periods of co-cultivation). Other substances, known to influence other monocyte or macrophage functions, such as prostaglandins, indomethacin, and corticosteroids, were without effect. Known inhibtors of superoxide or hydrogen peroxide generation had no effect on this assay in the dose ranges tested. This assay has been applied to patients with cancer, including those undergoing therapy with BCG or interferon. No statistically significant differences between patients undergoing immunotherapy and untreated patients or normal individuals have been established, although data analysis is still in progress.

A new microassay system for GIA, which allows drastic reduction in the number of cells required for its performance, has been validated in terms of reproducibility and statistical norms. Using cryopreserved effector cells as well as cryopreserved target cells has permitted minimization of day-to-day variation in the assay. Comparison with a large body of data obtained in the usual "macro" system showed excellent correlation with data obtained in this "mini" system.

Growth inhibition was also assayed in a system using inhibition of tumor cell line alkaline phosphatase (AP). This system gave results comparable to those obtained using tritiated thymidine incorporation, but also demonstrated novel findings. Monocytes apparently inhibit alkaline phosphatase activity directly since diminished activity was observed prior to changes in cell number or tritiated thymdine incorporation. When tumor cell lines without measurable alkaline phosphatase activity were co-cultured with monocytes and a cell line with high levels of the enzyme, monocyte inhibitory activity was diminished, suggesting competitive inhibition by targets sharing similar recognition structures. Thus, this assay may be quite useful to analyze in detail the nature of the determinants conferring sensitivity to GIA by monocytes. This observation could not have been made when tritiated thymidine incorporation was used as an index of cell number, since all cell lines incorporate thymidine at similarly high rates.

Monocyte function was also assayed by measuring cytotoxicity against tumor cell lines by a variety of isotope release assys. Using either ^{51}Chromium or ^{111}Indium release, monocytes had demonstrable cytotoxicity against K562 cells which could be inhibited by agents which interfere with phospholipid methylation, as had been demonstrated for NK cells. Recently developed assays for measuring ^{125}IUDR release from a varitey of adherent breast, kidney, fibroblast and glioma cell lines have not as yet been employed in extensive studies.

III. Monoclonal antibodies to cell surface components.

An enzyme-linked immunosorbent (ELISA) assay procedure developed by us was used to identify clones producing monoclonal antibodies to human monocytes or NK cell surface components. Mice were immunized with highly purified human mononuclear cells obtained by elutriation, in the case of monocytes, or by Percoll density gradient separation, for natural killer cells (large granular lympho

cytes, LGL). Spleens from immune mice were fused with mouse myeloma cell lines by the polyethylene glycol technique. Growing colonies of these fusion products with antibody activity specific for the immunizing cells and not human histocompatibility antigens were selected for further cultivation and cloning. Analysis of clones identified by the ELISA technique with the fluorescence-activated cell sorter or binding assays using indicator antibodies coupled to red blood cells are in progress to determine the specificity of these antibodies. Maintenance of clones with verifiable activity specific for these minor cell surface antigens remains a persistent technical problem.

Significance to Biomedical Research and the Program of the Insititute:

The place of lipid metabolism in immune regulatory and effector function and the role of monocytes in human defense against malignancy are both relatively unexplored areas of immunologic investigation. We have combined our interests in both these areas.

Phospholipids are components of the cell membrane which in large part determine its functional state. Changes in lipid metabolism result in profound effects on the interaction of the cell with its environment. We are attempting to gain an understanding of how agents which affect the immune system may mediate their effects by effecting changes in phospholipid metabolism.

Monocytes have been shown to play a significant role in controlling tumor cell proliferation in experimental animals and humans. Furthermore, a number of immunoadjuvants have been shown to exert their action via effects on the monocyte. Cytotoxicity and cytostasis represent in vitro correlates for in vivo functions of monocytes which are active against neoplastic cells.

The ability to distinguish monocyte or lymphocyte (NK)subpopulations by way of monoclonal antibodies could facilitate selection, cultivation, or enhancement of a desired function, or discrimination of undesired reactivities (i.e., suppression). A specific antigenic marker for NK cells or monocyte subpopulations would allow enumeration ofthese in the blood or other organs in a fashion similar to that used successfully to identify T cell subsets. The identificiation of antigens unique to cells in different stages of differentiation or development would also facilitate an understanding of the biologic role of NK cells and monocytes in tumor immunity.

Proposed Course of the Project:

More complete characterization of the interplay of different aspects of phospholipid metabolism is being undertaken. Specifically, the role of other pathways involved in metabolized phosphatidylcholine, including reacylation ("Lands pathway") and phospholipase C activity, will be evaluated in both NK cells and monocytes. The study will eventually be expanded to include metabolism of other lipids including phosphatidylinositol, neutral lipids, and ceramides and will examine their interaction with protein and sugar moieties of the cell membrane as well.

Further tests of monocyte function in normal individuals and patients with malignancy will be carried out, using the assays described above. The focus of

the study will be on examining the response to immunotherapy, (BCG, interferon, interferon-inducers) of patients with defined clinical status and immune capabilities. Simultaneously the mechanisms responsible for these effector functions such as superoxide and hydrogen peroxide generation will be examined.

Characterization of monoclonal antibodies selected by current screening procedures will be completed. Complement-mediated depletion of specific antigen-bearing cells and subsequent assay in standard functional tests (lymphoproliferation in response to mitogens and antigens, cytolysis and cytostasis, or suppression) will be attempted utilizing those antibodies of appropriate class or subclass specificity. Attempts will also be made to enumerate the representation of subpopulation-specific antigens in normal individuals and patient populations, and to correlate these with known markers (e.g. Fc or complement receptors, histocompatibility antigens such as Ia-like determinants, or antigens recognized by monoclonal reagents developed in other laboratories). Immunochemical analysis will be undertaken, in collaboration with nearby laboratories specialzing in chromatography and sequencing of membrane proteins, should such monocyte or subpopulation specific antigens be discovered.

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LABORATORY OF IMMUNOBIOLOGY

SUMMARY REPORT

October 1, 1980 to September 30, 1981

INTRODUCTION

Members of the Laboratory of Immunobiology continue to study immunologic mechanisms that impede tumor growth and spread in vivo and in vitro. The results of these studies have served as a basis for the design of clinical protocols to test the efficacy of immunotherapy for patients suffering from malignant melanoma or from head and neck cancer. In the Office of the Chief, Laboratory of Immunobiology, studies on the immunotherapy of cows with natural squamous cell carcinoma of the eye are being phased out. The decision to discontinue this service-contract supported investigation of "cancer eye" was based on the recommendation of the Board of Scientific Counselors of the Division of Cancer Biology and Diagnosis and of a special committee convened to evaluate the merits of the bovine cancer project. While some members of the special committee were favorably impressed by the rationale and potential utility of the "cancer eye" investigation, the decision of National Cancer Institute officials was to terminate the project. The service-contract supported project on the induction of malignant melanoma in guinea pigs is in its last year. The decision to end the guinea pig melanoma project was based on the conclusion that it would not fulfill the aim which was to provide a convenient model for the evaluation of immunoprophylaxis and immunotherapy in the control of experimental malignant melanoma.

In the Cellular Immunity Section studies have continued on attempts to establish effective immunotherapeutic methods for experimental cancer beyond stage I. We have succeeded in curing guinea pigs of stage II cancer by a combination of surgery and active specific immunization. The method is effective in eliminating occult lymph node metastasis as well as tumor intravenously implanted in the lungs. A vaccination procedure has been developed that eliminates dermal tumors and microscopic draining lymph node metastasis without the need for surgery. Intralesional injection of a mixture of emulsified trehalose 6,6-dimycolate and synthetic muramyl dipeptide was effective in causing tumor regression and elimination of lymph node metastasis. A lysozyme digest of a peptidoglycan from a Gram positive bacterium, Brevibacterium divaricatum could substitute for muramyl dipeptide. Evidence has been obtained indicating that a tumor rejection antigen shared by two chemically induced murine tumors may be coded for by endogenous murine leukemia virus. Adoptive immunity transfer from hyperimmunized donors to naive recipients appears to be mediated by a population of immune splenocytes that are distinct from those that kill the tumor cells in vitro. This conclusion was based on specificity tests and on immunoabsorption. Adoptive tumor immunity appears to require no host response and the transferred cells are randomly distributed in the skin. Microscopic foci of tumor cells in the lungs of mice established by intravenous injection could be eliminated by vaccination with mycobacteria or certain of their components. A model for breast cancer has been established in rats. Rats with the primary mammary adenocarcinomas induced by a single injection of a carcinogen can be treated successfully by immmunotherapy or surgery.

In the Humoral Immunity Section, mechanisms of complement activation by immunoglobulins on cell surfaces were studied. The immunoglobulins used in these studies were naturally occurring antibodies in humans and other species. One of these antibodies reacted specifically with methotrexate and another with folinic acid. Methotrexate and folinic acid covalently linked to sheep red blood cells bound specific immunoglobulin in different forms. The form in which an immunoglobulin was bound to the hapten on the cell surface determined whether the first component of complement became bound and if so whether as a result of binding it was activated. Evidence was found indicating that the mechanism of complement fixation differed for different classes of immunoglobulins. Studies were continued on the influence of lectins on complement action in the presence or absence of immunoglobulins. Some lectins can initiate activation, while others require the presence of immunoglobulin. Studies continued on the characterization of the reaction between protein A and immunoglobulins of various species and classes. It was concluded that in addition to the class of immunoglobulin, acquisition of a biological activity by the complex resulting from the interaction of protein A and immunoglobulin, depended on the size, molecular composition, distribution and density of the epitopes. Susceptibility of line 10 guinea pig hepatoma cells to immune lysis was ascribed in part to lipid synthesis. In contrast, the susceptibility of two human lymphoid cell lines to immune lysis could not be attributed to lipid synthesis. Lytic susceptibility did not correlate with membrane fluidity. The ninth component of complement is being separated from serum in order to provide a reagent that will facilitate studies on the mechanisms of immune cell killing.

In the Immunopathology Section studies are continuing on mechanisms of macrophage activation for tumoricidal activity. Development of tumoricidal macrophages occurs after a series of reactions each of which requires the simultaneous presence of an effective lymphokine signal and a macrophage intermediate responsive to that signal. Macrophages from certain strains of mice fail to develop tumoricidal activity after any of several treatments over a wide range of experimental conditions. The reasons for this lack of activity are not the same in every defective mouse strain. Lymphokine that regulates tumoricidal activity of macrophages is not the same as lymphokines that regulate certain microbicidal effects. Two steps have been found in the sequence of reactions leading to production of tumoricidal macrophages; one is a priming signal and the other a triggering signal. Detailed kinetic analyses of macrophage activation in vitro have revealed a reproducible time course of events. Macrophage activation for tumoricidal activity is associated with alterations in cell lipid content. In chemotaxis studies with human monocytes it was found that only a fraction of the total monocytes was capable of responding to attractant; this occurred without a lag period. Mouse macrophages differed in their behavior by showing a lag period and the percentage of responsive macrophages increased with cell concentration. Migrating monocytes were separated from non-migrating monocytes. Migrating monocytes were capable of binding attractant while non-migrating monocytes were not. By contrast non-migrating neutrophils were capable of binding as much attractant as the migrating population.

I. CELL MEDIATED ERADICATION OF TUMORS

A. Active specific immunotherapy

Studies have proceeded along two major paths: evaluating efficacy of active specific immunotherapy in guinea pigs with different stages of malignant disease and

modifying vaccines in an attempt to improve immunotherapeutic potency. We previously reported that immunization of guinea pigs with a vaccine containing irradiated tumor cells and an oil-in-water emulsion of BCG CW was effective in eradication of microscopic lymph node metastases remaining after surgery for clinical stage 1 experimental cancer. This year we discovered that this vaccine was effective in treatment of guinea pigs with more advanced malignant disease. Malignant disease produced in guinea pigs by intradermal inoculation of line-10 was allowed to progress to stage 2 at which time the dermal tumor and the first draining lymph node were grossly evident. At that stage the external appearance of the next draining lymph node was normal but it contained tumor cells. Limited surgery consisting of excision of the dermal tumor and first draining lymph node was not curative; palpable metastases developed in the second and other draining lymph nodes and at autopsy some animals were found to have grossly visible lung metastases. Immunization of guinea pigs with a mixture of irradiated syngeneic tumor cells plus mycobacterial cell walls in an oil in water emulsion eradicated tumor cells remaining in lymph nodes after limited surgery for stage 2 experimental cancer and prevented the progression of the disease to stage 3. Tumor intravenously implanted in the lungs of animals after limited surgery for stage 2 disease was also eliminated by immunization.

To provide a more rigorous test of vaccine potency, guinea pigs with a large tumor burden (both established dermal tumors and microscopic lymph node metastases) were treated by active specific immunotherapy. The vaccination procedure effective in treatment of guinea pigs with metastases remaining after surgery of stage 1 cancer was not effective in this disease setting. Successful vaccination required injection of a mixture containing irradiated tumor cells and heat killed whole mycobacterial cells.

B. Intralesional immunotherapy with natural and synthetic analogs of bacterial components

Mycobacterial components and analogs were tested for antitumor activity and toxicity. The antitumor activity of an emulsified mixture of trehalose 6,6'dimycolate and synthetic muramyl dipeptide was studied in guinea pigs each with an established syngeneic dermal tumor and microscopically detectable metastases in regional lymph nodes. A single intralesional injection of the emulsified mixture was an effective treatment causing tumor regression and elimination of lymph node metastases. A lysozyme digest of a peptidoglycan obtained from supernatant fluids of penicillin treated cultures of a Gram positive bacteria, Brevibacterium divaricatum NRRL-2311 could substitute for muramyl dipeptide.

C. Tumor rejection antigens of chemically-induced murine fibrosarcomas

We investigated the nature of a common tumor rejection antigen(s) in chemically-induced murine fibrosarcomas. Two methylcholanthrene-induced fibrosarcomas, previously demonstrated to contain a common tumor rejection antigen(s), released infectious ecotropic murine leukemia virus and expressed the murine leukemia virus proteins, gp70 and p15(E). To determine whether an antigen(s) specified by a murine leukemia virus might serve as a common tumor rejection antigen(s), primary cultures of syngeneic embryo cell cultures or an allogeneic cell line

were infected with an endogenous ecotropic murine leukemia virus obtained from one of the cross-reacting fibrosarcomas; expression of infectious and/or viral proteins was monitored and correlated with the results of transplantation protection tests. Uninfected allogeneic embryo cells (SC-1) did not release infectious virus or the viral protein gp70; mice immunized with SC-1 cells did not inhibit tumor growth. Uninfected syngeneic embryo cells did not release infectious virus but did release ug quantities of gp70 into supernatant fluids; mice immunized with uninfected syngeneic cells inhibited tumor growth in 2 of 7 experiments. Virus-infected syngeneic and allogeneic embryo cells released both infectious ecotropic murine leukemia virus and gp70; mice immunized with virus-infected cells inhibited tumor growth in 11 of 11 experiments. Growth of the two cross-reacting fibrosarcomas was inhibited in mice immunized with virus-infected embryo cells. The results indicate that antigens coded for by endogenous murine leukemia virus may function as common tumor rejection antigens on chemically-induced murine fibrosarcomas.

D. Studies of adoptive transfer of tumor immunity

Adoptive transfer of spleen cells from specifically immunized donors to non-immunized recipients was used to study tumor immunity in vivo to the syngeneic line-10 guinea pig hepatoma. Hepatoma cells cultured as monolayers on fibronectin coated surfaces served as targets for immune splenocytes in a ^{3}H release cytotoxicity assay in vitro. An antigenically distinct syngeneic guinea pig hepatoma was used to study the specificity of adoptive transfer of immunity and of the cytotoxicity in vitro. The protection afforded by adoptive immunization against challenge with hepatoma cells was tumor line specific, while in most cases cytotoxicity in vitro was not. The in vitro cytotoxic effect was abolished after absorption of the immune spleens with monolayers of either line-10 or line-1. In contrast, the in vivo tumor rejection activity of line-10 immune spleen cells was depleted after absorption with line-10 but not with line-1 or other control monolayers. These studies revealed that the immune cells mediating cytotoxicity in vitro were functionally distinct from those conveying adoptive protection in vivo.

Adoptive immunity was analyzed by a two dimensional titration of immune effector cells and tumor cell targets. A logarithmic plot of the two variables that produced 50% endpoints of tumor growth revealed a slope of 2.52. Increase in resistance to tumor challenge was an exponential function of increase in numbers of transferred effector cells. A two-fold increase in effector cells led to a 5.6 fold increase in resistance to tumor challenge. Adoptive transfer of immunity was independent of both secondary systemic and local immune responses. The distribution of tumor target cells had a major influence on the outcome of adoptive transfer of tumor immunity; a dose of effector cells that was unable to eradicate a given dose of tumor cells at one site was able to eradicate that dose distributed among multiple cutaneous sites.

E. Efficacy of mycobacterial components or irradiated tumor cells in treatment of mice with pulmonary metastases

Mice with microscopic foci of tumor cells in the lung established by intravenous injection of fibrosarcoma cells were treated with vaccine containing irradiated tumor cells alone, trehalose 6,6'dimycolate (TDM) alone, or mixture of irradiated tumor cells and TDM. Optimum conditions for therapy were determined. Intraperitoneal injection of irradiated tumor cells was effective in preventing the growth

of pulmonary tumor deposits. Mixtures of TDM and irradiated tumor cells were not more effective than irradiated cells or TDM alone. The therapeutic effect of the vaccine was immunologically specific since immunization of mice with an immunologically distinct fibrosarcoma failed to prevent the outgrowth of tumor cells. Cured mice were resistant to a challenge with living tumor cells.

The effectiveness of each of two mycobacterial components and a synthetic analog of one of them in the eradication of pulmonary deposits of intravenously injected syngeneic fibrosarcoma 1023 in C3H mice was studied. BCG cell walls (BCG CW), trehalose 6,6'-dimycolate (TDM) or 6,6'-di-O-2 tetradecyl, 3-hydroxyoctadecanoyl-, trehalose (C76), a synthetic analog of TDM, were administered in emulsified form by three different routes: intraperitoneal, intradermal, or intravenous, twenty four hours after intravenous injection of 1023 tumor cells. The most effective form of therapy was TDM given by the intraperitoneal route; about fifty percent of treated animals were cured. Higher doses of BCG CW or C76 also led to a significant number of cures. Each agent caused a significant prolongation of survival time of the treated mice, at two or more of the dosages tested; however, their routes of optimal activity varied.

F. Immunotherapy of primary breast adenocarcinomas in Buffalo rats

Three doses of the carcinogen n-nitroso n-methyl urea were tested for their capacity to produce malignant breast tumors by intravenous injection to female Buf/N rats. Rats received a single intravenous dose of NMU of 1.5, 2.5 or 3.75mg/100g body weight. At the lowest dose, 3% of carcinogen-treated rats developed breast tumors; none developed multiple primary tumors. At the intermediate dose, 19% of treated rats developed single breast tumors; 7% developed more than one primary breast tumor. At the highest dose, 30% of treated rats developed a single primary breast tumor; 30% of treated rats developed multiple primary tumors. Tumor bearing rats were treated by surgery or by intralesional injection of oil emulsions containing BCG CW. Surgery was effective treatment in most instances. BCG CW resulted in the regression or arrest of the tumor in a significant proportion of the animals. In untreated animals tumor grew progressively and metastasized to the lungs.

II. COMPLEMENT MEDIATED KILLING OF TUMOR CELLS

The role of immunoglobulins (Igs) in activating the complement (C) sequence has been further elucidated. The discovery in this laboratory that sera of several mammalian species (including man) contain significant levels of naturally occurring anti-methotrexate (MTX) and anti-folinic acid (FA) IgM antibodies facilitated these investigations. Advantage was taken of the fact that the haptens MTX and FA can be covalently complexed to sheep red cells and such labeled cells can be lysed by anti-MTX (or anti-FA) antibody and complement. Methods were developed to determine the number of haptens bound to the cell surface and the number of IgM molecules attached to the cells carrying different amounts of the hapten. It was found that the naturally occurring human IgM anti-MTX and anti-FA antibodies bound to cells in three different forms: one binds the first component of complement (C1) and activates the complete lytic sequence; the second binds C1 with no subsequent activation of the lytic sequence; and the third form neither binds C1 nor activates the lytic sequence. The ratio of the three forms depended on the density of haptens on the cell surface.

From these studies it was concluded that the binding of IgM to the cell by one antigen reactive site of the IgM molecule is sufficient to bind C1 but not sufficient to activate the lytic sequence. These observations may explain the basis of the controversy that exists among immunologists some of whom claim that monovalent antigen in combination with IgM antibodies could fix C; if the C fixation test was sensitive to the removal of C1, then C fixation would have been observed without consumption ("fixation") of any of the remaining C components in the sequence. Closely connected to the questions of the mechanism of C fixation by antibodies is the whereabouts of the bound C components relative to the location of the activating immunoglobulin molecule. Claims have been made that the fourth component of C (C4) in addition to the cell surface bound to the Fab portion of the IgG and that the C4 bound to IgG was the only kind of C4 participating in the lytic sequence. No such studies have been carried out with IgM except to show that IgM cold agglutinin was capable of fixing C4 to the cell surface and that removal of such IgM left C4 on the cell. Studies in this laboratory showed that C4 bound to the cell via IgM-C1 participated in the hemolytic reaction; that all IgM could be removed without removing any C4 and that transfer of IgM from cell to cell occurred without the transfer of C4. It was concluded that in contrast to IgG, no C4 was bound to IgM and that the hemolytic sequence was completed at a distance from the IgM molecule. These observations point to a fundamental difference in the mechanism of C fixation by different classes of Igs.

Efforts also continued to elucidate the mechanism whereby lectins initiate, augment or inhibit activation of C in the presence or absence of Igs. Succinylated and acetylated concanavalin A (Con A), but not the native lectin, activated the lytic C sequence on sheep cells in the absence of Igs. The effect was specific since succinylated wheat germ agglutinin was inactive and hemolysis was inhibited selectively by alpha-D-methylglucopyranoside. In related experiments it was found that native tetravalent Con A increased the lytic efficiency of C activated by anti-Forssman IgG. Other lectins with the same or different sugar specificity either augmented or inhibited hemolysis. Augmentation by Con A was consistent with the ability of the lectin on the cell surface to bind but not activate C1. Thus cell bound Con A and IgG under conditions where IgG by itself could not activate the lytic C sequence appeared to yield a complex that behaved like an aggregate of IgG molecules. These observations suggested the hypothesis that since Con A by itself could not activate bound C1, activation depended on the IgG in the complex. The identity and/or density of the antigen on the cell surface was also an important determinant for lectin induced augmentation of C activation. High doses of Con A inhibited by 80% the hemolytic activity of IgG antibodies against either a sugar-free antigen (human serum albumin) or an antigen reactive with Con A (human myeloma IgE) using cells to which these antigens were coupled by chromic chloride. Characterization at the molecular level of the reaction between protein A of Staphylococcus aureus and Igs of various species and classes also continued. The enhancement of the reaction between protein A and affinity purified rabbit IgG to human serum albumin (a polyvalent antigen) was correlated with immune aggregation but not with antigen induced allosteric changes in the Ig molecule. Absence of enhancement by immune complexes prepared with the monovalent hapten MTX and anti-MTX antibodies was consistent with the inability of these complexes to undergo aggregation. Similar results were obtained when antibodies and immobilized antigens were used to quantify the binding reaction between protein A and IgG. At least two complexes with different affinities could be formed as a function of the density of the immobilized ligand. From all of these studies the general conclusion was drawn

that for the acquisition of a biological activity by Igs, in addition to the class of Ig, the size, molecular composition, distribution and density of the immobilized ligand is of crucial significance. Whether the activated, potentially cytolytic C sequence can kill a cell depends in addition to all the above mentioned factors on the target cell itself. Pretreatment of guinea pig tumor cells with certain metabolic inhibitors, enzymes and X-irradiation increased while pretreatement with certain hormones decreased their susceptibility to killing by C. These effects were in general ascribed to the synthesis and composition of cellular lipids and other physiological properties of the cell. In attempts to determine if these observations were valid only for guinea pig hepatoma cells similar studies were performed using two human lyphoid cell lines, PY and Raji and the mouse mastocytoma cell line P815. These cells show variable susceptibility to C mediated attack during different parts of their growth curve. Cell cycle specific populations of cells did not demonstrate any variations in their susceptibility to C killing. It was also demontrated that selected chemotherapeutic agents rendered these cells susceptible to C killing while some hormones either had no effect or rendered PY and Raji cells more suseptible to C killing. As with the guinea pig tumor cells no correlation was found between the synthesis of cellular DNA, RNA, protein, complex carbohydrate and to susceptibility to attack by C; in contrast to the guinea pig cells susceptibility or resistance so far did not correlate with lipid synthesis. When cells at low density were cultured in media obtained from any phases of the growth curve or in fresh medium they became susceptible to killing by C within 4-18 hours of culturing. In contrast, cells at high density became susceptible only when cultured in fresh or log phase medium. So far none of these differences could be ascribed to changes in membrane fluidity as determined by flourescent polarization studies. These studies support the original observations that physiological properties of cells determine their susceptibility to killing by C; however, the actual controlling mechanisms still need to be further defined.

To elucidate the mechanism whereby C kills cells, C9, the component that generates the cell membrane lesion must be available in high purity and concentration. Methods are therefore being developed for the purification of C9 from whole human serum. Combinations of conventional separation techniques such as gel filtration and ion exchange chromatography have proved unsatisfactory for this purpose due to the similarity in charge and molecular weight characteristics between C9 and major serum constituents e.g. albumin and transferrin. The triazinyl dye Cibacron Blue 3G-A immobilized on cross-linked agarose has been found to bind all of the components of the human classical pathway which can then be eluted without substantial albumin contamination by the application of a linear salt gradient. Using this procedure C9 recoveries in excess of 100% (due to the possible removal of some inhibitor of C9 activity) and substantial increases in the specific activity of this component were possible. The product still contained other functionally active complement components, which were difficult to remove by conventional techniques. Recently a number of other triazinyl dye-matrix combinations have become available which show different binding affinities to those of Cibacron Blue. The interaction between four of these dyes and the components of the human complement pathway has been investigated with the view to their use in removing the contaminants from the C9 preparation. Whole serum applied to two of the matrices (Amicon Green A and Amicon Red A) was depleted of all nine components while there was no binding to either Amicon Blue B or Orange A. Bound components could be recovered

using linear concentration gradients of sodium chloride. The recovery profile from Green A was similar to that obtained with Cibacron Blue, although the increase in the specific activity of the eluted components was lower, due to the poorer total protein binding capacity of this matrix. In contrast, only three components could be eluted from Red A; C9, C1 and C4. C9 was recovered with a good yield (108%), and an increase in specific activity. Serum albumin could be removed by chromatography of the C9 pool from Red A on Cibacron Blue. Remaining contaminants, notably C4, could be removed by precipitation of a C9-rich fraction from whole serum with polyethylene glycol prior to chromatography on the Red A matrix.

III. IMMUNOLOGICAL INTERACTIONS OF HOST AND TUMOR

A. Macrophage Activation. Activated macrophages, cells with nonspecific tumoricidal and microbicidal activities, are recovered from animals with certain chronic infections such as Mycobacterium bovis, strain BCG for months after inoculation. However, regulatory mechanisms for induction and control of macrophage activation are operative for only a relatively brief interval, hours. Populations of activated macrophages that develop at sites of chronic infection or other chronic immune responses persist only because of continued presence of initiating antigens and constant replacement of lymphocyte-derived immune stimuli and responsive mononuclear phagocytes. Development of nonspecific effector function by activated macrophages during immune responses occurs after a series of reactions, each of which requires the simultaneous presence of an effective lymphokine signal and a macrophage intermediate responsive to that signal. The requirement for a reaction cascade, similar to blood coagulation or complement-mediated hemolysis, forms the basis of control mechanisms for regulation of macrophage effector function.

We have analyzed control of macrophage nonspecific effector function by several approaches:

(1.) Macrophages from certain strains of mice fail to develop nonspecific tumoricidal activity after any of several in vivo or in vitro treatments over a wide range of experimental conditions. Peritoneal macrophages from BCG-infected C3H/HeJ or A/J mice do not lyse any of a variety of tumor target cells; equal numbers of cells from BCG-infected C3H/HeN mice were strongly cytotoxic. Although the tumoricidal defects of C3H/HeJ and A/J cells appear phenotypically similar, each is mediated by distinct and genetically different control mechanisms.

Macrophages from BCG-infected but not from untreated or irritant-treated C3H/HeJ mice bind a variety of tumor cells as well as BCG-activated macrophages from the normal C3H/HeN strain. In contrast to C3H/HeN cells, however, macrophages from BCG-infected C3H/HeJ mice fail to secrete at least two activities that are directly cytotoxic to tumor cells: tumor necrosis factor and a neutral serine protease, cytolytic factor. Secretion of these soluble cytotoxic factors by macrophages from BCG-infected A/J mice was entirely normal. However, macrophages from this defective mouse strain exhibited a 75% decrease in ability to bind tumor cells. Thus, deficits in tumoricidial activity by cells from BCG-infected C3H/ HeJ or A/J mice result from mechanistically different defects in macrophage-tumor cell interaction.

(2.) Regulation of nonspecific macrophage effector function is mediated by several different lymphokines. For example, lymphokine activity for induction of macrophage tumoricidal function elutes from Sephadex G-100 as a single peak in the 50,000 mw region; lymphokine activities for induction of microbicidal effects against Rickettsia tsutsugamushi or Leishmania tropica elute in three different regions: 140,000, 50,000 and <10,000 daltons. Despite the apparent homogeneity of lymphokine activity for induction of macrophage tumoricidal effects, at least two functionally different signals can be identified. Macrophages respond to one mediator (priming signal) to enter into a noncytotoxic but highly receptive state in which they can then respond to a second lymphokine stimulus (trigger signal) to develop tumoricidal activity. Priming signals are effective at very low concentrations (1/500 dilution of active leukocyte culture supernatant) and and require 4 hours for optimal response; trigger signals are effective only at high concentrations (1/20) of the same supernatant but require less than 10 minutes for optimal response. It is the trigger signal that is limiting during immune reactions. Lymphokine priming and trigger signals form the basis of a regulatory system that sets the threshold and determines the onset of macrophage effector function.

(3.) Development of tumoricidal activity by macrophages treated in vitro with lymphokines follows a reproducible time course: cytotoxic activity is evident by 2 to 3 hours, becomes maximal by 6 to 12 hours and then progressively decreases to control levels by 24 hours. Loss of tumoricidal activity with time is not due to cell death or exhaustion of lymphokine. There are several major alterations in cell lipid content that correlate with the time for onset and loss of tumoricidal activity. These lipid alterations were evident 2 hours after lymphokine treatment; maximal changes occurred by 4 to 6 hours and progressively returned to initial values by 16 to 24 hours. At the maximum, total cell lipid increased 3 to 5 fold; cholesterol and triglyceride levels increased 5 to 10 fold. Saturated fatty acids in untreated macrophages comprised about 90% of the total fatty acid. In contrast, 75% of the fatty acid content of lymphokine-activated macrophages was unsaturated.

Activated macrophages from mice treated with viable BCG or killed Corynebacterium parvum show changes in lipid and fatty acid content similar to lymphokine-treated cells. Moreover, macrophages from C3H/HeJ mice respond to lymphokines by the criteria of increased glucose consumption but do not become tumoricidal; lymphokine-treated C3H/HeJ macrophages fail to develop the characteristic changes in lipid or fatty acid composition. Thus, macrophage activation for nonspecific tumoricidal activity in vivo and in vitro is associated with alterations in cell lipid content.

B. Leukocyte Chemotaxis. In a quantitative study of chemotactic responses of blood monocytes from normal human subjects, we found that only a fraction of the total monocytes migrated at optimal attractant concentration and incubation time. The size of this responsive subpopulation was between 25 and 40% of the total, depending on the donor, and was not affected by various alterations of in vitro conditions. In particular, when the input monocyte number was changed over a hundred-fold range, the percentage of migrating monocytes remained constant. Furthermore, when monocytes were added to chemotaxis chambers, they responded to attractant without a lag, time course curves being convex to the time axis with migrated monocytes increasing monotonically until a plateau was reached. Thus the migrating subpopulation of human monocytes was responsive to chemoattractant at the moment of addition to the chamber and the response appeared to be a simple interaction of individual cells with

chemoattractant, leading to directional movement. A similar analysis of mouse resident peritoneal macrophages revealed a remarkably different picture in two respects: the size of the responsive population increased with time, and the percentage of input macrophages that migrated toward attractant increased as the number of cells added to the chamber was increased. The time lag before significant chemotaxis responses occurred was shown by harvesting chemotaxis chambers at different times. A typical result with macrophage input numbers of 60,000 per well was migration of only 1000 cells during the first hour, whereas 24,000 migrated between 2 and 3 hours. Effects of cell concentration were shown in experiments with optimal chemotaxin concentrations and incubation times: only 3% of the macrophages migrated when the input number was 15,000 per well, whereas an average of 30% migrated at an input number of 120,000. Thus, responsiveness of mouse macrophages appears to be affected by cell-cell interaction on the chemotaxis membrane surface, and at the higher cell concentrations there is extensive cell-cell contact. The resident peritoneal cell population comprises lymphocytes, macrophages and mast cells. Lymphocytes and mast cells were separated from macrophages by filtration on Sephadex G-10 columns. Addition of the lymphocyte-mast cell population to fixed numbers of unseparated peritoneal cells caused an increase in the percentage of macrophages responding to chemoattractants.

As noted above, only 20-40% of human blood monocytes migrate to chemoattractants and that each of these migrating cells can respond to several different attractants. Many experimental approaches were used to determine if the 60-80% non-migrating population was due to an artifact of the in vitro system, and none was found. The validity of the approach was further susbstantiated by studies on chemotaxis of cultured mouse bone marrow cells. Chemotactic activity reached a peak between days 11 and 14 of culture, with 70 to 100% of the input cells migrating. In addition to the intrinsic biological interest, the result shows that low chemotactic responses of other cells are not due to limitations of the measuring system.

Comparison of the characteristics of migrating and non-migrating leukocyte populations was made possible by the design of a separation chamber, a modification of the 48-well chemotaxis chamber. There are 4 large cell input compartments in the separation chamber, so that after the chemotaxis incubation the non-migrated cells can be aspirated from the top of the chamber and the migrated cells can be washed off the attractant side of the chemotaxis membrane. A by-product of this approach is that it provides the only available method for obtaining pure, functionally active monocytes. The purpose of the study was to compare chemoattractant binding to migrating and non-migrating populations of human monocytes. Cells were incubated with the radioactive chemotactic peptide, F-met-leu (^{3}H) phe. Binding to the migrated population was saturable at room temperature, and 50% of maximal binding occurred at $10^{-8}M$, the concentration that induced optimal chemotaxis. Nonmigrating monocytes did not bind peptide under the same conditions, showing that at least one reason for non-responsiveness to chemotaxin is apparent lack of receptors. It was shown previously that exposure to superoptimal levels of chemoattractant caused subsequent unresponsiveness to that attractant without impairment of response to a different attractant (specific deactivation). Chemotactic deactivation of human monocytes with $5x10^{-7}M$ F-met-leu-phe reduced the number of available binding sites by 60%. Detection of receptor appearance and disappearance may provide a tool to investigate monocyte maturation and biochemical mechanisms of chemotaxis. For example, does absence of peptide receptors on the the non-migrating human

monocytes reflect a maturational stage of the cells? The question can now be approached experimentally. In this context, the results of similar studies on human neutrophils are of great interest. The non-migrating neutrophil population had the same binding affinity and receptor number for F-met-leu-phe as the migrating population. Thus, the deficiency in the human neutrophil non-migrating population appears to be subsequent to receptor-ligand binding.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08525-05 LIB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunotherapy of Primary Autochthonous Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. Rapp	Chief, Laboratory of Immunobiology	LIB NCI
OTHER:	J. T. Hunter	Expert	LIB NCI
	S. Sukumar	Visiting Fellow	LIB NCI
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COOPERATING UNITS (if any)

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3.0	1.5	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mammals with naturally occurring or experimentally induced primary autochthonous cancer are being studied as guides to the immunotherapy of human cancer. It is hoped that these guides will provide information more relevant to the treatment of human cancer than that derived from studies with transplanted tumors. Cattle with ocular squamous cell carcinoma ("cancer eye") have been treated successfully by the injection of BCG cell wall vaccine directly into the tumor. Primary autochthonous tumors of rats, and humans are also being studied.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The primary objective of this project is to test a variety of therapies in several species with primary cancer in the hope of providing guides to the immunotherapy of human cancer. The therapeutic methods tested will be those that have been successful in animal models consisting of syngeneic tumor transplants.

Methods Employed: The primary autochthonous cancers studied were either naturally occurring or were induced by chemicals. Natural primary cancers of cows and of humans and chemically induced primary cancers in inbred rats were treated by intratumoral administration of living BCG or emulsified components of BCG.

Hereford cattle with histologically confirmed ocular squamous cell carcinoma and with no palpable lymph nodes were treated by intratumoral injection of emulsified BCG cell walls, local surgery or radical surgery. Survival of treated animals was compared to that of animals receiving sham vaccine or no treatment.

Human patients with cancer in the region of the head and neck are being treated in a prospective randomized trial by intratumoral injection of emulsified BCG cell walls followed by standard surgical treatment. The rate of recurrence of disease and of survival of the patients treated by preoperaive immunotherapy are being compared to that of patients treated by surgery alone.

In a previous prospective randomized study, patients with Stage I malignant melanoma but at high risk of disease recurrence within five years after surgery were treated by the intratumoral injection of living BCG followed by standard surgical treatment. The rate of disease recurrence and survival of the patients treated by preoperative immunotherapy are being compared to that of patients treated by surgery alone.

Breast cancer was induced in rats by a single intravenous injection of N-nitroso N-methyl urea into approximately 50 day old animals. A dose of carcinogen was used that produced malignant disease in about 30 percent of the animals. A majority of animals with breast cancer developed only a single lesion. Animals with primary autochthonous breast cancer were treated by intralesional BCG emulsion or by surgery.

Major Findings: Statistical analysis of the results of the trial of intralesional BCG for bovine ocular squamous cell carcinoma permitted the conclusion that the group of animals treated by intratumoral injection of emulsified BCG survived significantly longer than controls.

The prospective randomized trial of immunotherapy for the treatment of humans with primary cancer in the head and neck region is in its fifth year. Fewer patients treated by preoperative immunotherapy have had recurrent disease or have died than have those treated by surgery alone. Patients treated by intratumoral injection of BCG cell walls experienced a mild, transient increase in body temperature but none of the severe complications seen in some patients treated by the intratumoral injection of living BCG.

The prospective randomized trial of immunotherapy for the treatment of patients with Stage I, high risk malignant melanoma has been terminated. The main reason for closing the trial was that some patients treated by intralesional injection of living BCG suffered a severe thrombocytopenia. All patients with BCG associated thrombocytopenia eventually recovered from this side effect. As of November 1977 thirteen patients were randomized to preoperative intralesional BCG and thirteen to surgery alone. Statistical analysis of the treatment results with a median follow-up of about 4 years indicates that intralesional BCG treatment can reduce recurrence frequency and increase the proportion of survivors following surgery of patients with Stage I primary melanoma. These conclusions need to be tested in a larger clinical trial of immunotherapy for patients with malignant melanoma but emulsified instead of living BCG should be used.

At autopsy untreated rats with primary breast cancer were found to have lung metastases. Intralesional BCG was effective treatment for rats with breast cancer A significant number of rats were benefited by this treatment. Tumors regressed completely in some animals and evidence indicated that BCG treatment prevented visceral metastases.

Surgery was also effective treatment. Tumors recurred in a significant number of animals treated with BCG or by surgery.

Significance to Biomedical Research and the Program of the Institute: Clinical trials of postoperative immunotherapy for humans with cancer have yielded disappointing results. Intratumoral injection of emulsified mycobacterial components either preoperatively or alone has been curative for guinea pigs with an intradermally transplanted, metastasizing hepatoma. This experimental finding suggested that intratumoral injection of mycobacterial components might be curative for hosts with primary autochthonous cancer. Results consistent with this suggestion have been found in the treatment of cattle with naturally occurring, primary, autochthonous, ocular squamous cell carcinoma. Two clinical immunotherapy trials, one for patients with primary head and neck cancer and the other for patients with primary malignant melanoma, have yielded sufficiently promising results to suggest the establishment of additional prospective randomized trials of immunotherapy consisting of the intratumoral injection of BCG cell walls.

The lack of therapeutic success, either by surgery or by intratumoral BCG, achieved in studies of rats with primary autochthonous chemically induced cancers of the digestive tract raised questions about the appropriateness of these models for experimental treatment studies. One difficulty with these models was the high frequency of multiple (probably primary) cancers in individual animals. Multiple primary cancers occur only rarely in human patients or in cows with ocular carcinoma.

Proposed Course of Project: Prospective randomized clinical trials of intratumoral BCG are in progress for humans with head and neck cancer. The malignant melanoma trial has been terminated and patients are being followed. Contract support for studies of bovine "cancer eye" has been withdrawn. Major effort will be expanded on development of the rat model of primary autochthonous breast cancer. This model will be used for basic studies of anti-cancer mechanisms and as a guide in the development of new treatments for breast cancer.

Rapp, H.J.: Immunotherapy of animals with primary induced cancer. Letter to the Editor, Cancer Immunology and Immunotherapy, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08526-05 LIB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunologic Approaches to the Treatment of Animals with Clinical Stage I Malignant Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	J. Hunter	Expert	LIB NCI
	M. P. Ashley	Visiting Fellow	LIB NCI
	T. Sugimoto	Visiting Fellow	LIB NCI
	E. Yarkoni	Guest Worker	LIB NCI

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to establish the conditions and methods best suited to the prevention and treatment of cancer by methods involving immunostimulation. The areas of current interest are 1) the determination of conditions required for immunologic eradication of lymph node metastases and 2) the study of the cellular and molecular basis of eradication of tumor cells within lymph nodes.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The principal objective of this project is to develop, in an experimental system, safe and effective vaccines to eradicate microscopic lymph node metastases remaining after surgical removal of a primary tumor.

Methods Employed: Model of clinical stage I disease with microscopic metastases. The line-10 transplantable hepatoma, derived originally from a primary hepatoma induced in a male strain-2 guinea pig by diethylnitrosamine, was maintained as an ascites tumor. Intradermal injection of line-10 cells into syngeneic guinea pigs (10^6 cells per guinea pig) produced dermal tumors with subsequent development of lymph node metastases.

Immunotherapy of animals with dermal tumors and micrometastases. Tumor cell-containing vaccines were tested for ability to cause regression of dermal tumors and to prevent growth of metastases. The vaccines contained live or irradiated line-10 tumor cells admixed with a mycobacterial adjuvant consisting of an emulsion of oil droplets containing BCG killed cells (BCG KC).

Major Findings: Eradication by active specific immunotherapy of established dermal tumors and microscopic lymph node metastases. Treatment with living line-10 tumor cells admixed with emulsified heat-killed Mycobacterium bovis strain BCG cells caused complete regression of established dermal tumors (about 10 mm in diameter) and prevented the growth of microscopic lymph node metastases in 25 of 39 treated animals (64%). Tumor growth at the vaccine site occurred in 2 of 39 treated animals. Treatment with irradiated line-10 tumor cells admixed with BCG cells caused complete regression of established dermal tumors and prevented the growth of microscopic lymph node metastases in 66 of 82 treated animals (80%). Growth of tumors at the vaccine site did not occur in animals that received vaccines containing irradiated tumor cells. Untreated animals, animals treated by intradermal injection of BCG cells alone or with irradiated tumor cells alone, all died with progressive tumor growth. The intradermal route of administration of the vaccine was immunotherapeutically more effective than the subcutaneous route. Emulsions that were immunotherapeutically active contained 3% squalane and 0.2% Tween 80 or 3% squalene and 2% vitamin E. Eradication of dermal tumors and microscopic lymph node metastases was dependent on both the dose of irradiated tumor cells and of mycobacterial cells. The minimum dose of mycobacterial cells that was effective was 900 μg; 2700 μg produced regression in virtually all treated animals. The minimum dose of irradiated tumor cells that was effective when administered with an optimal dose of adjuvant was about 10^7 cells; 10^8 cells produced regression in virtually all treated animals. Killed whole mycobacterial cells or mycobacterial cell walls functioned effectively as an adjuvant. Vaccines were effective when administered either in one or three quadrants.

Significance to Biomedical Research and the Program of the Institute. The results of these experiments confirm our previous studies on the efficacy of active specific immunotherapy for treatment of guinea pigs with the line-10 tumor. The vaccine described in this report is remarkable since it is effective in causing regression of established tumors and preventing the growth of microscopic metastases. Analysis of the factors essential for the success of active specific

immunization in this guinea pig model will serve as a valuable guide in the design of vaccines for treatment of guinea pigs with more advanced stages of malignant disease and for treatment of animals with primary autochthonous cancers.

Proposed Course of Project: Rats with chemically-induced breast cancer and cattle with ocular squamous cell carcinoma will be treated by active specific immunotherapy. Studies will be performed to elucidate the basis of active specific immunotherapy.

Publications

Ashley, M.P., Zbar, B., Hunter, J.T., Rapp, H.J., and Sugimoto, T.: Adjuvant-antigen requirements for active specific immunotherapy of microscopic metastases remaining after surgery. Cancer Res. 40: 4197-4203, 1980.

Yarkoni, E. and Rapp, H.J.: Regression by active specific immunotherapy of established dermal tumor implants and lymph node metastases in guinea pigs. Infect. Immun. 31: 514-516, 1981.

Ashley, M.P., Zbar, B., Hunter, J.T., Sugimoto, T., and Rapp, H.J.: Post-surgical treatment of microscopic lymph node metastases with vaccines containing tumor cells and BCG cell walls. In Proceedings of "Prospects for Manipulation of Host-Tumor Relationships", Blois, France, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08527-05 LIB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunologic Approaches to Treatment of Animals with Clinical Stage II Malignant Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	M. Ashley	Visiting Fellow	LIB NCI
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COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to establish the conditions and methods best suited to the prevention and treatment of cancer by Immunostimulation. The area of current interest is immunotherapy of animals with microscopic lymph node metastases or occult visceral metastases remaining after surgery.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The primary objective of this project is to define the methods and conditions best suited for immunotherapy of clinical stage II malignant disease.

Methods Employed: Tumor. A transplantable syngeneic cavian hepatocellular carcinoma, designated line-10, was used.

Cavian model for clinical stage II malignant disease. Line-10 cells were injected intradermally into strain-2 guinea pigs. When the draining lymph nodes were palpable, animals were treated by excision of the dermal tumor and superficial distal axillary lymph node followed by active immunotherapy. Animals were immunized two days after surgery with vaccines containing live or irradiated tumor cells admixed with an oil-in-water emulsion of BCG CW.

Murine model for treatment of microscopic pulmonary metastases. C3H/HeN MTV male mice were injected by the i.v. route with Tumor 1023 cells (10^5). Mice were treated 1 day after injection of tumor cells by i.d., i.p., or i.v. injection of trehalose dimycolate (TDM), BCG cell walls (CW) or X-irradiated Tumor 1023 cells.

Major Findings: Treatment by limited surgery and specific immunization of guinea pigs with stage II experimental cancer. When palpable lymph node metastases developed in the SDA lymph node, tumor cells were present in the PA lymph nodes and to a lesser extent in more distant lymph nodes. Though some degree of anti-tumor immunity was generated at or after the time of challenge, it was evidently insufficient to prevent the progressive growth of micrometastases remaining after limited surgery.

Preparations containing irradiated L10 and BCG CW were reproducibly effective for treatment of animals with residual stage 1 disease and were not tumorigenic. This approach was used to treat PA lymph node metastases remaining after limited surgery for stage II disease with the difference that two treatments were given: the first on the ipsilateral flank followed 1 week later by a booster treatment on the contralateral flank. This treatment prevented growth of residual lymph node metastases in 29 of 38 animals treated. In the groups treated by surgery alone (excision of the primary tumor and SDA lymph node) metastases in the PA node grew in all but 2 of the 30 animals, and more than half of the animals had pulmonary metastases at death.

The efficacy of limited or extensive surgery with or without active specific immunization was evaluated in animals with clinical stage II disease, with and without the added burden of pulmonary tumor deposits implanted i.v. on the day of surgery. In animals with regional disease and no i.v. implanted tumor: a) All animals treated with limited surgery died with extensive lymph node metastases and a majority had grossly evident lung metastases. b) Extensive surgery cured a significant proportion of the treated animals. c) Limited surgery plus active immunization also cured a significant proportion of treated animals. Active specific immunotherapy plus limited surgery was no more effective than extensive surgery. In animals with regional disease and i.v. implanted pulmonary tumor: a) Animals treated either by limited or extensive surgery died. b) A significant proportion of animals treated by active immunization after limited or extensive surgery survived.

Murine model for treatment of microscopic pulmonary metastases: The effectiveness of each of two mycobacterial components and a synthetic analog of one of them in the eradication of pulmonary deposits of intravenously injected syngeneic fibrosarcoma 1023 in C3H mice was studied. BCG cell walls (BCG CW), trehalose 6,6'-dimycolate (TDM) or 6,6'-di-O-2 tetradecyl, 3-hydroxyoctadecanoyl-α, α trehalose (C76), a synthetic analog of TDM, were administered in emulsified form by three different routes: intraperitoneal, intradermal, or intravenous, twenty four hours after intravenous injection of 1023 tumor cells. The most effective form of therapy was TDM given by the intraperitoneal route; about fifty percent of treated animals were cured. Higher doses of BCG CW or C76 also led to a significant number of cures. Each agent caused a significant prolongation of survival time of the treated mice, at two or more of the dosages tested; however, their routes of optimal activity varied.

Mice with microscopic foci of tumor cells in the lung established by intravenous injection of fibrosarcoma cells were treated with vaccines containing irradiated tumor cells alone, trehalose 6,6'dimycolate (TDM) alone, or mixtures of irradiated tumor cells and TDM. Optimum conditions for therapy were determined. Intraperitoneal injection of irradiated tumor cells was effective in preventing the growth of pulmonary tumor deposits. Mixtures of TDM and irradiated tumor cells were not more effective than irradiated cells or TDM alone. The therapeutic effect of the vaccine was immunologically specific since immunization of mice with an immunologically distinct fibrosarcoma failed to prevent the outgrowth of tumor cells. Cured mice were resistant to a challenge with living tumor cells.

Significance to Biomedical Research and the Program of the Institute: Control of residual malignant disease remains one of the foremost problems in human cancer therapy. The therapeutic efficacy of surgery and active immunization for animals with stage II and III disease offers some hope that analogous treatment may be effective for humans with stage III cancer provided that human malignant neoplasms contain appropriate antigens. The hope for therapy of stage III disease must be tempered by the fact that in this and other studies, pulmonary tumor deposits were introduced at a time when animals were at clinical stage I or stage II and, therefore may have been in a better state of health than would have been animals with stage III disease arising as a consequence of the dermal implant.

Proposed Course of Project: Animals with microscopic visceral metastases will be treated by active specific immunotherapy.

Publications

Hunter, J.T., Ashley, M.P., Sukumar, S., Sugimoto, T., Zbar, B., Rapp, H.J., and Yarkoni, E.: Treatment by limited surgery and specific immunization of guinea pigs with stage II experimental cancer. J. Exp. Med., in press.

Sukumar, S., Hunter, J.T., Yarkoni, E., Rapp, H.J., Zbar, B., and Lederer, E.: Efficacy of mycobacterial components in the immunotherapy of mice with pulmonary tumor deposits. Cancer Immunol. Immunother., in press.

Hunter, J.T., Ashley, M.P., Rapp, H.J., Sukumar, S., and Zbar, B.: Pulmonary metastases in guinea pigs as a consequence of dermal implantation of line-10 tumor cells. Cancer Immunol. Immunother., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08528-05 LIB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanism of Delayed Hypersensitivity and Tumor Graft Rejection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to identify the nature of tumor transplantation rejection antigens on chemically-induced tumors. The current area of interest is the nature of common tumor rejection antigen(s) in chemically-induced murine fibrosarcomas.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The goal of this project is to define the nature of cross-reacting tumor rejection antigens on chemically-induced murine fibrosarcomas.

Methods Employed: Animals. Male C3H/HeN MTV-mice were obtained from the Charles River Laboratories, Wilmington, Mass.

Tumors. Transplantable syngeneic fibrosarcomas, designated 1023, 1038 and 1063 were induced in the subcutaneous tissues of C3H/HeNIcr male mice with pellets of 1% 3-methylcholanthrene in paraffin. Tumor cell suspensions were prepared by enyzmatic digestion of minced tumors.

Preparation, infection and maintenance of syngeneic and allogeneic embryonic cell cultures. Fetuses, removed aseptically from mice, were disaggregated by incubation of minced fetal tissue with trypsin. Cell cultures were established by seeding disaggregated embyro cells in tissue culture flasks. Cell cultures were infected with murine leukemia virus (MuLV) twenty-four hours after cells were seeded by replacement of the existing nutrient medium with medium containing polybrene and MuLV. Nutrient fluids were replaced 24 hours after infection and thereafter at 2-3 day intervals. Seven - eight days after cells were seeded, the cells were harvested and placed in new tissue culture flasks. Cells, harvested from tissue culture flasks on day 14, 15, or 16, were washed once and resuspended in medium without serum or antibiotics. Cells were kept on ice until just before injection into syngeneic mice.

SC-1 cells, a clonal line of wild mouse embryo, were grown in Eagles minimal essential medium with 10% fetal bovine serum. Virus infected or control uninfected SC-1 cells were used for immunization of C3H/HeN mice.

Immunization. Mice were immunized by i.p. injection of syngeneic or allogeneic embryo cells infected with MuLV or not infected with MuLV.

Quantitation of host resistance. To determine the degree of resistance produced by immunization, control and immunized mice were challenged with graded doses of tumor cells; for each dose of tumor cells 5-10 animals were tested. The number of cells required to produce tumors in 50% of mice were calculated according to the Pizzi formula. The RI was the difference between the $\log_{10}$TD50 in immunized and normal animals. Significance of the difference between values of $\log_{10}$TD50 in different groups of animals was estimated by Student's t-test. When the value of t was greater than or equal to 2.0, the difference between the $\log_{10}$TD50 in the two compared groups of animals was considered significant. The response of immunized and control mice was also evaluated with the Mantel-Haenszel test.

Major Findings: Expression of MuLV by murine fibrosarcomas and by MuLV-infected mouse embryo cells. We measured the release of infectious ectropic MuLV by the XC test and the expression of structural antigens of MuLV by reacting cells with a panel of monoclonal antibodies. The fibrosarcomas released different quantities of infectious, ecotropic MuLV. Tumor 1063 released about 600 fold more virus than Tumor 1038; Tumor 1023 did not release infectious MuLV. Results of the infectious center XC plaque assay correlated both with results of electron microscopic examination and with tests for virus on supernatant fluids of cultures of tumor cells.

Each tumor line expressed all p15(E) epitopes; the profile of expression of epitopes of ecotropic gp70 varied from tumor to tumor.

All tests for MuLV expression on uninfected SC-1 cells were negative. Uninfected syngeneic embryo cells did not release infectious virus or react with monoclonal antibodies to ecotropic MuLV, but did release a gp70 immunologically related to gp70 molecules of leukemia viruses capable of infecting heterologous species cells (xenotropic MuLV). This conclusion was based on the results of immunoaffinity chromatography and tryptic peptide map analysis. Syngeneic or allogeneic embryo cells infected with MuLV 1063c released infectious virus and reacted with all 8 monoclonal antibodies. Cells infected with the recombinant MuLV, AKR 13 MC, were XC test negative, did not react with antibodies to gp70, but did react with monoclonal antibodies to p15(E).

Systemic resistance to tumor challenge after intraperitoneal immunization with MuLV infected syngeneic or allogenic embryo cells. Uninfected allogenic embryo cells (SC-1) did not release infectious virus or the viral protein gp70; mice immunized with SC-1 cells did not inhibit tumor growth. Uninfected syngeneic embryo cells did not release infectious virus but did release μg quantities of gp70 into supernatant fluids; mice immunized with uninfected syngeneic cells inhibited tumor growth in 2 of 7 experiments. Virus-infected syngeneic and allogeneic embryo cells released both infectious exotropic murine leukemia virus and gp70; mice immunized with virus-infected cells inhibited tumor growth in 11 of 11 experiments. Growth of the two cross-reacting fibrosarcomas was inhibited in mice immunized with virus-infected embryo cells. The results indicate that antigens coded for by endogenous murine leukemia virus may function as common tumor rejection antigens on chemically-induced murine fibrosarcomas.

Significance to Biomedical Research and the Program of the Institute. Tumors induced by chemical carcinogens have been thought to contain individually specific tumor rejection antigens. Recently, reports have appeared that document examples of cross protection among respiratory tract carcinomas in rats, cutaneous squamous cell carcinomas in mice and carcinogen induced fibrosarcomas in mice. The results of our experiments suggest an explanation for these examples of cross protection. Antigens coded for by endogenous retroviruses may serve as tumor rejection antigens on tumors induced by chemical carcinogens. It may be possible to immunize the host to these antigens and prevent chemical or physical carcinogenesis. Other investigators have shown that leukemia in AKR mice and leukemia induced by X-irradiation in C57BL/6 mice can be prevented by passive immunization with antibodies to MuLV.

Proposed Course of Project: We will attempt to identify the tumor rejection antigen(s) present in syngeneic mouse embryo cells infected with MuLV.

Ecotropic, dual-tropic and amphotropic MuLV, as well as replication defective MuLV, will be used as probes to determine which viral antigen(s) are necessary to produce resistance to a challenge with a chemically-induced fibrosarcoma.

We will correlate results of quantitative analyses of tumors for content of infectious MuLV and of viral antigens with susceptibility to immunoprophylaxis. Emphasis

will be placed on tumors induced by ultraviolet light since some investigators have reported that these tumors contain a common TRA.

Experiments will be performed to confirm and extend the observations of Huebner and coworkers who demonstrated that active immunization to MuLV interferes with chemical carcinogenesis.

Publications

Zbar, B., Sugimoto, T., Manohar, V., and Ashley, M.P.: Immunoprophylaxis of transplantable methylcholanthrene-induced murine fibrosarcomas by immunization with syngeneic embryo cells infected with murine leukemia virus. In Proceedings of "Prospects of Manipulation of Host-Tumor Relationship", Blois, France, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08529-05 LIB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunotherapy with Defined Bacterial Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. Rapp	Chief, Laboratory of Immunobiology	LIB NCI
	E. Yarkoni	Guest Worker	LIB NCI
OTHER:	M. P. Ashley	Visiting Fellow	LIB NCI
	B. Zbar	Chief, Cellular Immunity Section	LIB NCI
	J. T. Hunter	Expert	LIB NCI
	T. Sugimoto	Visiting Fellow	LIB NCI
	S. Sukumar	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

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Laboratory of Immunobiology

SECTION

Cellular Immunity Section

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1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Components of mycobacteria injected in oil/water emulsions into syngeneic tumors growing in the skin of guinea pigs or mice caused the tumors to regress. Animals cured of their tumors were resistant to rechallenge with the same tumor.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The main objective of this project is to identify mycobacterial components with maximal antitumor effects and minimal toxicity.

Methods Employed: Malignant hepatoma designated line 10 was induced in strain-2 guinea pig by the feeding of diethylnitrosamine. Ascites variant of the tumors was maintained by serial intraperitoneal passage. Oil-in-water emulsions containing bacteria or mycobacterial components were prepared by ultrasonication or by grinding. The following bacteria or their components were investigated: Live Mycobacterium phlei bacilli, heat killed M. phlei bacilli, heat killed Nocardia rubra bacilli, heat killed BCG bacilli, trehalose-6,6'-dimycolate (TDM) and muramyl dipeptide (MDP).

Emulsions containing bacteria or bacterial components were infiltrated into the growing tumors. The tumors were subsequently observed for progression or regression.

Major findings: The antitumor activity of a mixture of synthetic N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) and trehalose-6,6'-dimycolate (TDM) (MDP+TDM) in emulsified form was studied in guinea pigs, each with a syngeneic dermal tumor and microscopically detectable metastases in regional lymph nodes. A single intralesional administration of an ultrasonically prepared emulsion containing MDP+TDM in squalane or in mineral oil caused tumor regression and elimination of lymph node metastases. Similar emulsions of MDP+TDM made with squalene or hexadecane were immunotherapeutically inactive.

Heat-killed cells of Mycobacterium bovis (BCG), Mycobacterium phlei and Norcardia rubra were each tested in emulsified form for their ability to cause regression of established dermal transplants and lymph nodes metastases of a syngeneic hepatocarcinoma in guinea pigs. On a weight basis, BCG was superior to N. rubra in causing tumor regression. Under the conditions tested N. rubra was inferior to M. phlei in its antitumor activity. M. phlei and BCG were approximately the same in their therapeutic potency. In BCG sensitized guinea pigs, N. rubra provoked a weaker delayed cutaneous hypersensitivity (DCH) reaction than did BCG. In N. rubra-sensitized guinea pigs, BCG provoked a weaker DCH reaction than did N. rubra. Purified protein derivative of M. tuberculosis was more active in eliciting DCH in BCG-sensitized guinea pigs than in animals sensitized with N. rubra.

Living M. phlei bacilli in aqueous suspension were able to cause regression of established dermal line 10 tumor transplants and lymph node metastases in guinea pigs.

Significance to Biomedical Research and the Program of the Institute: Immunotherapy with intralesionally administered mycobacterial components was effective treatment for animals with solid tumors and with no evidence of gross lymph node or visceral metastasis. There are clinical situations in which similar treatment might be a practical and desirable approach. Killed BCG with TDM or M. smegmatis cell wall skeletons with TDM have been reported to be effective in the treatment of human skin cancer. Nonviable mycobacterial components could provide stable and easily standardized immunotherapeutic agents and would

avoid the risk of progressive infection. Several nonviable mycobacterial preparations have been found to be at least as effective as living BCG for experimental tumor immunotherapy if the agents are administered intralesionally. Additional studies to establish the safety and efficacy of such agents for clinical use are in progress. Toxicity studies in experimental animals could identify immunotherapeutic doses of mycobacterial components that produce acceptably low levels of hypersensitivity reactions. The availability of synthetic compounds for cancer immunotherapy offers an opportunity to explore the relationship between the chemical structure of the agent and its tumor regressive potency. The results of such studies may permit the design of molecules with antitumor activity and without unwanted side effects.

Proposed Course of Project: Experiments designed to determine the requirements for successful immunotherapy will be continued. Emphasis will be placed on the isolation and definition of additional components of mycobacteria which cause regression of dermal tumors and palpable lymph node metastases. Synthetic analogs of mycobacterial components will be investigated for their antitumor activities. Attempts will be made to cure advanced tumors, which currently available mycobacterial preparations failed to affect, by a combination of chemotherapy and immunotherapy.

The optimal doses, and kinds of drugs and mycobacterial preparations and schedules of administration will be established. Efforts will be made to prepare vaccine(s) composed of attentuated tumor cells and mycobacterial preparation(s) for the effective treatment of animals with visceral metastasis.

Publications

Yarkoni, E. and Rapp, H.J.: Regression by active specific immunotherapy of established dermal tumor transplants and lymph node metastases in guinea pigs. Infect. Immun. 31: 514-516, 1981.

Yarkoni, E., Rapp, H.J., and Hunter, J.T.: Treatment of mice with lung metastases from a dermally, implanted fibrosarcoma: Comparison of intratumoral trehalose-6,6' - dimycolate (cord factor) and surgery. Eur. J. Cancer 17: 291-295, 1981.

Yarkoni, E., Lederer, E., and Rapp, H.J.: Immunotherapy of experimental cancer with a mixture of synthetic muramyl dipeptide and trehalose dimycolate. Infect. Immun. 32: 273-276, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08530-02 LIB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immune Eradication of Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. Rapp	Chief, Laboratory of Immunobiology	LIB	NCI
	S. Shu	Expert	LIB	NCI
OTHER:	J. T. Hunter	Expert	LIB	NCI
	L. Fonseca	Guest Worker	LIB	NCI

COOPERATING UNITS (if any)
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SECTION
Cellular Immunity Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were carried out on adoptive tumor immunity in naive guinea pigs that received specifically sensitized spleen cells from hyperimmunized donors. The effector spleen cells were also tested for their capacity to kill the tumor target cells in vitro. Tumor resistance in vivo increased experimentally as a function of the number of intravenously transferred sensitied spleen cells. Within the tumor challenge doses analyzed, suppression of tumor growth by adoptive immunity appears to be independent of specific immune response in the recipient. Studies on distribution and expression of antitumor immunity after intravenous infusion of immune cells revealed that the transferred immunity was distributed randomly throughout the skin of the recipient and there was no evidence indicating that they are attracted specifically to the tumor inoculation site.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The principal goal of this project is to study the mechanisms of immunological eradication of a syngeneic tumor by sensitized lymphoid cells. Since lymphoid cells capable of transferring antitumor immunity interact with but fail to lyse tumor targets in vitro, we have analyzed the underlying mechanisms exclusively by adoptive transfer.

Methods Employed: Adoptive transfer of antitumor immunity. Spleen cells from strain-2 guinea pigs immunized to line-10 hepatoma were injected by the intravenous route into normal guinea pigs and each recipient received a single or multiple intradermal challenge with line-10 tumor cells. Suppression of tumor growth at skin challenge site provides a means to evaluate transferred immunity.

Mathematical and statistical methods. TD_{50} defined as tumor growth suppression in 50 percent of test animals was calculated by Reed-Muench formula and the standard error was computed by Pizzi's formula. The TD_{50} correlation between the number of immune spleen cells and tumor challenge dose was evaluated by linear regression analysis with the aid of a programmed calculator (HP-97, Hewlett-Packard Co.). Significance of differences between TD_{50} was estimated by Student's t-test.

Major Findings: As indicated in last year's progress report, we demonstrated by an absorption procedure that immune lymphoid cells capable of conveying adoptive antitumor immunity reacted with but failed to lyse tumor targets in vitro. These findings led us to conclude that data from adoptive transfer may be required in order to form a basis for mechanistic studies of in vivo antitumor immunity. Therefore we have conducted a series of adoptive transfer experiments in which the quantitative relationship between immune lymphoid cells and tumor challenge doses was analyzed. The highlights of the analysis may be summarized as follows: 1) The number of tumor cells eradicated increased exponentially as a function of the number of immune lymphoid cells transferred; a two-fold increase in transferred immune cells led to a 5.6 fold increase in resistance to tumor challenge. 2) Eradication of 10^4 or 10^5 line-10 tumor cells by adoptive immunity was independent of specific immunity in the recipient. Therefore, adoptive transfer under defined conditions may be used as a quantitative assay for the antitumor activity of immune lymphoid cells. 3) Rejection in 50 percent of test animals of a 10^4 line-10 cell challenge in the presence and absence of 99 x 10^4 irradiated tumor cells required 18 and 25 x 10^6 immune lymphoid cells respectively. This finding indicated that local immune stimulation did not significantly contribute to the rejection of 10^4 line-10 cells by adoptive immunity. 4) Transfer of 100 x 10^6 immune cells to naive receipients was not sufficient to reject 10^7 line-10 cells located at one skin site, but was sufficient to reject 10^7 tumor cells inoculated at multiple (10) skin sites. In another experiment, the rejection of a 10^6 line-10 challenge by adoptive transfer of immune spleen cells was not influenced by the presence in a single site of 10^7 live line 10 cells. Thus the transferred immune cells appeared to be distributed randomly throughout the skin of the recipient. There was no evidence indicating that the immune cells were attracted specifically to the tumor challenge site. 5) The number of immune cells required to suppress the growth of a 10^5 line-10 challenge when

admixed in the tumor inoculum was about 70 times less than when given systemically. This finding together with the results of the study on skin distribution of intravenously transferred immune cells suggests that only a small proportion of transferred immune cells participates in tumor suppression at a challenge site.

Significance to Biomedical Research and the Program of the Institute: The recent development of methods that permit replication in vitro of T lymphocytes that retain immunological reactivities has led to renewed interest in the use of adoptive immunity as a means of specific immunotherapy of cancer in man. Findings summarized in our experiments not only contribute to our understanding of the nature and characteristics of interactions between immune cells and tumor targets in vivo but also provide a quantitative foundation for clinical application of adoptive immunotherapy of cancer.

Proposed Course of Project: Logical extension of this project includes: 1) identification of host components (cellular and/or humoral factors) that contribute to or influence the efficacy of adoptive immunity, 2) study of the significance of host specific immunity to adoptive antitumor immunity in immunologically compromised animals, 3) the role of regional lymphs node in the induction of specific antitumor immunity.

Publications

Shu,S., Hunter, J.T., Rapp, H.J., and Fonseca, L.S.: Adoptive Immunity to a Syngeneic Guinea Pig Hepatoma: Characteristics of Effectors and Quantitative Analysis of Tumor Rejection. In The Potential Role of T Cell Subpopulations in Cancer Therapy. New York, Raven Press, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08550-07 LIB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Modification of Tumor Cells and Immune Cytolysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	A. Mitsuoka	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pretreatment of tumor cells with chemotherapeutic agents, metabolic inhibitors, enzymes or hormones modifies the susceptibility of the cells to killing by antibody plus complement. The purpose of this investigation is to determine the attributes of cells which influence the cells' ability to modify immune attack and to determine the molecular aspects of C-mediated killing of nucleated cells by immune attack.

Project Description

Objectives: 1) To study the mechanism whereby enzymes, metabolic inhibitors and anti-lipidemic agents increase the sensitivity of tumor cells to immune attack; 2) to study mechanisms whereby hormones decrease sensitivity of cells to immune attack; 3) to determine metabolic pathways and the physical and chemical properties of the cell that may be modified following such treatment; 4) to determine the cellular processes that may be modified following immune attack; 5) to study the molecular aspects of antibody-C lysis of nucleated cells.

Methods Employed: Antibody is quantitated by the complement (C) fixation and transfer test and immune cytolysis. Sensitivity of cells to antibody-C attack is measured by uptake of trypan blue and/or release of ^{125}IUdR. Incorporation of radioisotopically-labeled precursors of DNA, RNA, protein, complex carbohydrate, glycoproteins, glycolipids and lipids are being used to measure general metabolic properties of the cells. Thin layer chromatography is being utilized to analyze and identify specific lipid moieties synthesized by the cells. Sucrose density gradient ultracentrifugation is used to prepare plasma membrane and intracellular membrane fractions of tumor cells. High pressure liquid chromatography (HPLC) is being utilized to determine the lipid and fatty acid content and composition of the cells. Sensitivity of cells to cell-mediated immune attack is measured by ^{51}Cr release. Immunochemical methods, including Sephadex and DEAE chromatography, electrophoresis, immunodiffusion and ultracentrifugation are employed to isolate and identify biological macromolecules.

Major Findings: The malignant guinea pig hepatoma cells, line-1 and line-10, are relatively more resistant to killing by antibody plus guinea pig C (GPC) than by antibody plus human C (HuC). Line-10 cells sensitized with anti-Forssman antibody are resistant to killing by GPC whereas line-1 is not, despite the fixation of equivalent quantitaties of antibody and the initial C components (C1, C4, and C3).

Cells pretreated with certain metabolic inhibitors or chemotherapeutic agents are rendered sensitive to antibody-GPC killing; cells pretreated with selected polypeptide, catecholamine, or steroid hormones are rendered resistant to antibody-HuC killing. The drug and hormone effects are time and concentration dependent and are reversible; the ability of the cells to resist antibody-C killing correlated with their ability to synthesize complex lipids, but not DNA, RNA, protein, or complex carbohydrate.

Metabolic inhibitors (adriamycin and actinomycin D) that increase the sensitivity of the cells to antibody-C killing were examined for their effects on the ability of the cells to synthesize and incorporate specific lipids into plasma membrane and intracellular membrane fractions. Cells that had been rendered sensitive to antibody-C killing were inhibited in their incorporation of newly synthesized phosphatidylcholine and cholesteryl ester into the plasma membrane, as well as incorporation of phosphatidylcholine, cardiolipin, cholesteryl ester, and triglyceride into certain intracellular organelles including mitochondria, endoplasmic reticulum, nuclear membrane, or microsomes. Drug-treated cells recultured in the absence of the drug regained their ability to resist antibody-C killing and to synthesize and incorporate lipids into plasma and intracellular membranes. These data suggested that agents modifying the sensitivity of the tumor cells to humoral immune killing have a concomitant effect on plasma membrane and intracellular lipid synthesis.

Line-10 cells that had been rendered resistant to antibody-C-mediated killing following incubation for 1 hr with insulin or hydrocortisone were enhanced in their incorporation of newly synthesized L-α-phosphatidylserine, L-α-phosphatidylcholine, and triglycerides into the plasma membrane as well as L-α-phosphatidylcholine, L-α-phosphatidylserine, and cholesteryl ester into mitochondria, endoplasmic reticulum, nuclear membrane, or microsomes; these ccells were inhibited in cardiolipin synthesis. Cells cultured for 4 hr with hormone regained their sensitivity to antibody-C-mediated killing and reverted to control levels in their ability to synthesize and incorporate lipids into plasma and intracellular membranes. These data suggest that agents that increase the resistance of the tumor cells to humoral immune killing stimulate the synthesis and incorporation of specific complex lipids into plasma membrane and intracellular organelles; these effects generally were opposite those observed after treatment with metabolic inhibitors.

Drug treatment reversibly increases, whereas hormone treatment reversibly decreases the polyunsaturated fatty acid content of the cellular phospholipids. Hormone treatment also increases the total polarity of cellular fatty acids. The drug and hormone effects on the lipid and fatty acid composition of the cell affect certain molecular relationships in the cells. Drug treatment decreases the cholesterol: phospholipid mole ratio and increases the unsaturated fatty acid content of cellular phospholipids and neutral lipids. Hormone treatment causes the opposite changes. The changes in these molecular relationships could have marked effects on certain physical properties of the cell, especially membrane fluidity, permeability, or thickness. Effects on the physical properties of the cells could explain the drug and hormone effects on the susceptibility of the cells to humoral immune attack.

Analysis of the lipid and fatty acid content of subcellular fractions from the drug- and hormone-treated cells showed that the changes in lipid composition observed in whole cell lipid extracts were a reflection of changes occurring in the plasma membrane as well as in intracellular membranes (i.e., mitochondria, nucleat membrane, endoplasmic reticulum, microsome). Adriamycin treatment decreased the unesterified cholesterol and saturated triglyceride content while increasing the polyunsaturated fatty acid-containing triglycerides and phospholipids in the plasma and intracellular membranes; 5-fluorouracil (a drug that is not effective in increasing the sensitivity of the cells to antibody-C killing) did not have these effects. Treatment of the cells with insulin or hydrocortisone had exactly the opposite effects of those observed in the adriamycin-treated cells. These results suggest that modifications in the chemical composition of intracellular membranes as well as the plasma membrane of nucleated cells may influence certain physical properties of the cell membranes (e.g., fluidity, permeability) and as a result, could affect the outcome of humoral immune attack at the cell surface.

Line-10 tumor cells cultured for 24 hr in lecithin-rich normal human plasma or with synthetic lecithin showed a 5- or 8-fold increase in their lecithin:sphingomyelin mole ratio without being affected in their total lipid content or cholesterol: phospholipid mole ratio. These cells were more sensitive to killing by antibody plus C than untreated controls. Line-10 cells that underwent a homogeneously catalyzed hydrogenation reaction were reduced 6-fold in their content of unsaturated fatty acid compared to controls; the lipid content of these cells was largely unaffected. These cells were more resistant to antibody-C-mediated killing than controls. These modifications in cellular lipid and fatty acid composition could be reversed when the cells recultured for 24 hr in serum-containing tissue culture

medium; the cells regained controls levels of susceptibility to antibody-C killing at this time. These results suggest that by manipulating the lipid or fatty acid composition of a tumor cell, either indirectly by changing the lipid composition of the environment in which the cell resides or by directly altering the chemical nature of a cellular lipid constituent, the susceptibility of the cell to humoral immune killing can be modulated.

Studies on the variation and modification of nucleated cells to antibody-C killing were extended to include human lymphoid cell lines PY and Raji and the mouse mastocytoma cell P815. Raji and PY in their lag or stationary phase of growth are relatively more resistant to killing by rabbit ALS or human anti-HLA plus C. The difference in sensitivity observed for the cell lines was not due to differences in antigen expression as measured by quantitative absorption test. Cells isolated in G_1 or S phase of the cell cycle were equally sensitive to antibody-C killing indicating the variation in sensitivity of the cells is not directly cell-cycle dependent.

The mouse mastocytoma cell line, designated P815, also demonstrated variation in sensitivity to antibody-C killing. These cells which were relatively more resistant at the log phase of growth expressed the least amount of antigen. The sensitivity of the line-1, line-10, the human lymphoblastoid and mouse mastocytoma cell lines to killing by C from different species was also determined. Human, rabbit and goat C were generally more effective in killing the guinea pig and human cells. Rat C was effective in killing line-1 cells sensitized with anti-Forssman or antitumor antibody was only effective in killing line-10 cells sensitized with antitumor antibody and slightly effective in killing human lymphoid cells in stationary phase of growth. With the mouse cell line P815, guinea pig was as effective as human and goat C; rat C was less effective and rabbit C the most effective in killing the cells sensitized with the heterologous rabbit anti-P815 antibody.

The sensitivity of the human cell lines and the mouse mastocytoma P815 to killing by antibody plus C can be increased following treatments with various metabolic inhibitors. The effectiveness of the drugs in rendering the human cells sensitive was dependent upon the growth phase of the cells in culture. Cells in stationary phase were generally not rendered susceptible to killing whereas cells in lag or log phase of growth could be rendered more susceptible than untreated control cells. The mouse mastocytoma cell lines appear to show the opposite. Cells in the stationary phase generally were rendered susceptible.

Studies on the effects of lipids on C-mediated killing of line-1 and line-10 tumor cells showed that phosphatidylglycerol, cardiolipin, phosphatidylethanolamine were effective in inhibiting the cytotoxic action by human, rabbit and goat C. Phosphatidylserine and phosphatidylglycerol were effective in inhibiting GPC activity while phosphatidylglycerol enhanced rat C activity against line-10 cells sensitized with antitumor antibody but not anti-Forssman antibody. The inhibitory effects of the various phospholipids are dependent upon antibody used to sensitize the cells on concentration of lipids used to treat the C and on the time the lipids were added to HuC. The inhibitory effect of the lipids appeared to be at the stage of formation of T*. Addition of lipids to T* did not interfere with its transformation to dead cells. T* is an intermediate in the killing of cells by antibody plus C and contains all the components of C required for cell killing to occur.

Under conditions of limiting C almost all lipids tested interfered with C activity. Additional studies indicated that the fatty acyl composition and subtle structural configuration of the lipids influenced C activity. Modification of C activity also occurred upon addition of free fatty acid to C. Enhancement or inhibition or no effect was observed and appeared to be dependent upon the fatty acid, fatty acid concentration, specificity of the antibody and concentration of C. Pretreatment with selected fatty acids, but not more complex lipids, increased the sensitivity of the cells to killing by C.

Analysis of membrane fluid properties were performed on the human and mouse cells at different parts of the cell growth cycle. Fluorescence polarization studies with cells labeled with the lipophilic fluorescent probe 1,6-diphenylhexatriene showed the membranes of the antibody-C resistant cells to be relatively more resistant than those of the sensitive cells. Resistant Raji cells cultured for 24 hr at a density (0.5×10^6 cells/ml in fresh media or media from log or stationary phase cultures became sensitive to antibody-C attack; membrane fluidity increased upon culture in fresh medium only. Resistant Raji cells cultured at high density (1.5×10^6 cells/ml) under similar conditions became sensitive only after culture in fresh or log phase media. No detectable change in membrane fluidity was observed. The fluid properties of cells treated with metabolic inhibitors either effective or not effective in rendering cells susceptible to antibody-C attack were generally greater than untreated control cells. These results suggest that physiological of nucleated cells are as important as physico-chemical properties in determining susceptibility in immune attack.

Significance to Biomedical Research and the Program of the Institute: Modification of a tumor cell by metabolic inhibitors, hormones, chemotherapeutic agents and anti-lipidemic agents, or through chemical or physical manipulation of the cell's macromolecular composition furnishes a new tool to study the interaction of tumor cells with the immune defense mechanisms of the host. The study of the response by tumor cells to humoral or cellular immune attack through modifications in various cellular metabolic pathways provides information regarding the mechanism of defense or cytomembrane repair processes in these cells. Modification of these processes may lead to cells that are more vulnerable to immune attack mechanisms.

Proposed Course of Project: Radioisotope incorporation studies will be continued as probes to determine the cellular functions that are modified by treatment with drugs or hormones. The chemical attributes of normal cells, tumor cells and treated tumor cells will be studied. Work will continue on the effect of enzymes, hormones and inhibitors of macromolecular synthesis on the metabolic function of the tumor cells. Analysis of membrane-associated and intracellular macromolecules in these cells will be pursued. Further quantitative chemical analysis of the lipid and fatty acid composition of cells that are susceptible or resistant to immune killing will be made. Other means of manipulating tumor cell macromolecule composition without directly affecting cellular metabolic pathways will be pursued.

The effect of antibody and C on nucleated cells will be further analyzed. This will include studies on the binding and utilization of C components during the cytotoxic reaction. The effects of the stages in the growth cycle of the cell and the physical and biochemical events that occur during formation and transformation of T* will also be studied.

Publications

Boyle, M.D.P. and Ohanian, S.H.: Evidence for the influence of the initial complement components on the assembly and activity of the membrane attack complex. J. Immunol. 124: 2824-2827, 1980.

Ohanian, S.H. and Schlager, S.I.: Humoral immune killing of nucleated cells: Mechanisms of complement-mediated attack and target cell defense. In Atassi, M.Z. (Ed.): CRC Critical Reviews in Immunology. Boca Raton, Fla., CRC Press, Vol. 1, 1981, pp. 165-209.

Schlager, S.I. and Ohanian, S.H.: Cellular membranes and host-tumor interactions. In Van den Bossche, H. (Ed.): The Host-Invader Interplay. Amsterdam, Elsevier/ North Holland, 1980, pp. 15-30.

Schlager, S.I. and Ohanian, S.H.: Modulation of tumor cell susceptibility to humoral immune killing through chemical and physical manipulation of cellular lipid and fatty acid composition. J. Immunol. 125: 1196-1200, 1980.

Schlager, S.I. and Ohanian, S.H.: Tumor cell lipid composition and sensitivity to humoral immune killing. II. Influence of plasma membrane and intracellular lipid and fatty acid content. J. Immunol. 125: 508-517, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08551-06 LIB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Induction of Tumor Immunity by Chemotherapy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Ohanian	Research Microbiologist	LIB NCI
OTHER:	T. Borsos	Chief, Humoral Immunity Section	LIB NCI

COOPERATING UNITS (if any)

Freie Universitat Berlin, Berlin, West Germany

LAB/BRANCH
Laboratory of Immunobiology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Selected chemotherapeutic agents injected into tumors growing in vivo cause the tumor to regress. Animals cured of their tumors are resistant to rechallenge with the same tumor. The purpose of this work is to analyze the mechanisms of intralesional chemotherapy and incude histological techniques, and methods for analyzing humoral and cellular mediated immune mechanisms.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: 1) To cure tumors and induce tumor immunity by direct treatment of established tumors with chemotherapeutic agents; 2) to study the development of cellular and humoral immunity in cured animals.

Methods Employed: Inhibition of tumor growth in animals cured of tumors by intratumoral chemotherapy is used to measure the development of tumor immunity. Intradermal or sucutaneous implantation of guinea pig and mouse tumors into syngeneic animals are used to determine malignancy. Immunological techniques employed to measure humoral and cellular immunity include complement fixation, antibody-complement-mediated killing and cell-mediated cytotoxicity. Histological and radioisotope procedures are used to determine the effect and distribution of chemotherapeutic agents.

Major Findings: Intralesional injection of selected chemotherapeutic drugs causes regression of the malignant guinea pig hepatoma line-10. Intralesional injections of actinomycin D, adriamycin, BCNU, cisplatinum, vincristine, and bleomycin were effective in causing tumor regression. Methotrexate, DTIC, and 5 FU are not effective in causing regression of tumor. Injections, starting 7 days after tumor implantation, were given 5 times a week for 3 weeks. At this time, metastasis to draining lymph nodes had already occurred. Subsequent experiments showed that single intralesional injection of drugs was as effective as multiple injections of the drugs in causing tumor regression. However, systemic injection of similar concentrations of the drugs into tumor-bearing animals were toxic.

Intralesional chemotherapy of 7 day intralesional line-10 tumor with one injection of mixtures of Atromid-S plus DTIC or plus 5 FU were not effective in causing tumor regression. Different concentrations of Atromid-S (300 and 600 mg/kg) were mixed with nontoxic concentrations of DTIC (20 mg/kg) or 5 FU (15 mg/kg). In in vitro studies, Atromid-S inhibited synthesis of lipids and 5 FU inhibited synthesis of DNA, RNA, protein and complex carbohydrate, but not lipid synthesis. No information is available on the effects of DTIC on macromolecular synthesis.

Histological studies following a single intralesional injection of adriamycin or DTIC into line-10 tumor were performed. Samples taken from the tumor site showed little host cell infiltration into the tumor site following injection of adriamycin compared to DTIC in saline-injected controls. The regional draining node of tumor-bearing animals was tumor-free within 7 days following intralesional injection of adriamycin. Minimal systemic effects were detected in adriamycin-treated animals as determined by differential analysis of blood and development of immunity in animals cured of their tumors.

Analysis of the persistence of drug following intralesional injection of ^{57}Co-bleomycin into tumor-bearing mice indicated that no difference in the rate of elimination following i.v., i.p., i.m., s.c., or i.t. injection from tumor-free or tumor-bearing animals with 5 day old tumors. Significant retarded elimination was observed in animals bearing 12 day old tumors. Differences in distribution of labeled drug into various organs was observed following injection by the different routes. In general, there was less of a systemic distribution when the drug was injected i.t., i.m., or s.c. and tumor-bearing animals showed a lower concentration in the organs compared with that of tumor-free animals. Neither after i.v. nor i.t. injection of ^{57}Co-bleomycin in the mice bearing 5 day old tumors do the

lymph nodes draining the tumor attain the levels of radioactivity detected in lymph nodes of normal mice or animals bearing 12 day old tumors.

Additional experiments have been performed in tumor-bearing mice and guinea pigs comparing the effectiveness of aqueous solutions of bleomycin with water-in-oil emulsions of bleomycin. Preliminary results indicate cures can be obtained with much lower concentrations of bleomycin in a water-in-oil emulsion than in water solution.

In attempts to determine if the host immune response influenced the effectiveness of chemotherapy tumor bearing guinea pigs were treated with rabbit anti GP ALS before and following intralesional chemotherapy with vincristine. ALS was found to prevent the cure of animals treated with low concentrations of vincristine. Animals treated with 10-fold higher concentrations of the drug were cured of their tumor but were not immune to challenge. Tumor-bearing animals treated with just the vincristine were cured and immune to challenge.

Significance to Biomedical Research and the Program of the Institute. These studies in a syngeneic tumor animal model system are designed for analyzing the mechanism of intralesional chemotherapy. The development of this sytem should make it possible to better utilize intratumoral therapy of human cancer.

Proposed Course of Project: Further studies on the effects of intratumoral chemotherapy into tumors growing for longer than 7 days. Additional histology of the tumor sites at various times after injection of chemotherapeutic agents will be studied. Studies will be performed to determine the effect of multiple small injections of drugs into tumors growing for longer than 7 days. Techniques of humoral and cellular immunity to the tumor will be employed to more critically define the immune status of the treated animals.

Publications

Ohanian, S.H., Schlager, S.I. and Goodman, D.: Analysis of the intralesional adriamycin-induced regression of primary and metastatic growth of line-10 guinea pig hepatoma. Cancer Immunol. Immunother. 8: 179-187, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08552-15 LIB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanism of Complement Fixation and Action

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Borsos	Chief, Humoral Immunity Section	LIB NCI
OTHER:	J. J. Langone	Expert	LIB NCI
	A. P. Gee	Visiting Fellow	LIB NCI
	R. Ejzemberg	Guest Worker	LIB NCI
	A. Circolo	Visiting Fellow	LIB NCI
	H. Kato	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Immunobiology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is a long-range project investigating the mechanism of complement fixation and action. In particular the interaction of antibody-antigen complexes with the first component of complement and the result of this interaction on the other components are investigated. The relation between antibody action and complement activation is also explored. Finally, the significance of complement in the humoral immune defense mechanism is studied.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To develop new methods and to use available complement (C) fixation tests based on the fixation and transfer of the first component of C (C1) and cytotoxic tests for the analysis of antigen-antibody reactions, in particular in the search for cancer specific antigens.

Methods Employed: The model for studying cytotoxic reactions mediated by antibody and C consists of sheep erythrocytes, hemolytic antibody and C. Purification procedures for antibodies and the C components include: preparative (large-scale) gel filtration, ion exchange chromatography and preparative free electrophoresis. Other techniques used include precipitin and immunoelectrophoretic analysis, analytical, zonal and preparative ultracentrifugation and other immuno- and physico-chemical methods. Development and application of quantitative analytical methods which utilize radiolabeled tracer molecules are being emphasized.

Major Findings: Most naturally occurring antibodies are immunoglobulins (Ig) of the IgM class. IgM antibodies bound to cell surface haptens may or may not "fix" complement. It has been postulated that C fixing properties of cell surface bound IgM antibodies depend on the number of antigen reactive sites bound in the IgM molecule. Following the discovery in this laboratory that most mammalian sera contain naturally occurring anti-methotrexate (MTX) and anti-folinic acid (FA) the C-fixing and activating properties of these naturally occurring antibodies was studied. Advantage was taken of the fact that these haptens can be covalently linked to sheep erythrocytes and that such labelled cells can bind the naturally occurring antibodies. Sheep erythrocytes carrying the hapten-IgM complexes can be lysed upon the addition of C. In studying the mechanism of C activation by these complexes it was discovered that the efficiency of lysis depended on the amount of MTX or FA used for labeling. Methods were then developed to determine the number of haptens bound to the cell surface and the number of IgM molecules attached to the cells carrying different amounts of the hapten. It was found that the amount of lysis obtained with a given amount of bound IgM was proportional to the amount of hapten bound to the cell. The conclusion was drawn that IgM antibodies bound to cell surfaces in three different forms: one that binds the first component of C with no subsequent activation of the lytic sequence; the second that binds C1 and activates the lytic sequence; and a third that does neither. The ratio of the three forms depended on the density of the haptens on the cell surface. A consequence of these findings was the conclusion that binding of IgM to the cell by one antigen reactive site may be sufficient to bind C1 but not sufficient to activate the lytic sequence.

Closely connected to the problem of mechanism of activation of the lytic sequence by Igs is the location of the bound C components relative to the activating Ig molecule. Several years ago members of this laboratory demonstrated that each IgM-C1 complex on the cell surface was capable of binding up to 60 molecules of C4 to the cell and that most of these could participate in the lytic sequence. Methods were developed to remove the IgM from the surface of the cells and it was found that no C4 molecule was removed under conditions when no measurable IgM was left on the cell.

The cell-bound C4 molecules were still capable of interacting with C2, the next component in the sequence, and were able to participate in the lysis of the cell. These observations demonstrated that the role of the IgM and of the C1

molecule in the lysis of cells was confined to binding of C4 and C2 to the cell and to generate the enzyme $C\overline{42}$ (C3 convertase).

Significance to Biomedical Research and the Program of the Institute: C fixation is one of the most widely used diagnostic tools. The development and successful application of a very sensitive C fixation test, the C1FT test, opened up new possibilities in determining antigen-antibody reactions on cell surfaces.

Furthermore, cytotoxic reactions due to antibody and C are prime examples of body defense mechanisms. Fundamental research into the nature and mechanism of C fixation and action will contribute greatly to the development of diagnostic tools and to the understanding of the mechanism of immune body defenses.

Proposed Course of Project: This is a long-range project, and little change is expected in the scope of the work during the next few years. The ultimate goals of this project are the development of better diagnostic tools and the elucidation of molecular events associated with the action of C and antibodies. It is hoped that as a result of our program of inquiry into the basic problem of the interaction of antibodies, antigens and components of C, tools will be developed that are of practical significance in the search for cancer antigens.

Publications

Borsos, T., Chapuis, R.M. and Langone, J.J.: Distinction between fixation of C1 and the activation of complement by natural IgM anti-hapten antibody: Effect of cell surface hapten density. Mol. Immunol., in press.

Borsos, T., Chapuis, R.M. and Langone, J.J.: Natural antibodies in mammalian sera to folinic acid and methotrexate. Mol. Immunol., in press.

Borsos, T., Loos, M., Chapuis, R.M., Medicus, R. and Isliker, H.: A novel way of relating the structure of C1q to the hemolytic activity of the first component of complement. Mol. Immunol. 17: 1415-1421, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08555-09 LIB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Detection of Complement Components on Nucleated Cell Surfaces

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Borsos	Chief, Humoral Immunity Section	LIB NCI
OTHER:	M. D. P. Boyle	Visiting Scientist	LIB NCI
	A. P. Gee	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Immunobiology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.75	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to analyze the mechanism of action of complement in the lysis of various cellular targets. Techniques will be developed to quantify, on a molecular basis, complement components bound to the cell membrane. The biochemical events leading to the generation of complement lesions will be studied.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To determine the factors governing the susceptibility of different targets to antibody-complement killing and 2) to develop techniques for the detection of complement (C) components bound to the cell surface.

Methods Employed: Immunochemical methods including DEAE chromatography, electrophoresis and immunodiffusion are employed to isolate antibodies, protein and C components. Quantitation of antibody and C components on a molecular basis is carried out using radioisotopic labeling, quantitative precipitin procedures, C fixation tests, and other immunochemical tests.

Major Findings: To elucidate the mechanism whereby C kills cells, C9, the component that generates the cell membrane lesion must be available in high purity and concentration. Methods are therefore being developed for the purification of C9 from whole human serum. Combinations of conventional separation techniques such as gel filtration and ion exchange chromatography have proved unsatisfactory for this purpose due to the similarity in charge and molecular weight characteristics between C9 and major serum constituents e.g. albumin and transferrin. The triazinyl dye Cibacron Blue 3G-A immobilized on cross-linked agarose has been found to bind all of the components of the human classical pathway which can then be eluted without substantial albumin contamination by the application of a linear salt gradient. Using this procedure C9 recoveries in excess of 100% (due to the possible removal of some inhibitor of C9 activity) and substantial increases in the specific activity of this component were possible. The product still contained other functionally active complement components, which were difficult to remove by conventional techniques. Recently a number of other triazinyl dye-matrix combinations have become available which show different binding affinities to those of Cibacron Blue. The interaction between four of these dyes and the components of the human complement pathway has been investigated with the view to their use in removing the contaminants from the C9 preparation. Whole serum applied to two of the matrices (Amicon Green A and Amicon Red A) was depleted of all nine components while there was no binding to either Amicon Blue B or Orange A. Bound components could be recovered using linear concentration gradients of sodium chloride. The recovery profile from Green A was similar to that obtained with Cibacron Blue, although the increase in the specific activity of the eluted components was lower, due to the poorer total protein binding capacity of this matrix. In contrast, only three components could be eluted from Red A; C9, C1 and C4. C9 was recovered with a good yield (108%), and an increase in specific activity. Serum albumin could be removed by chromatography of the C9 pool from Red A on Cibacron Blue. Remaining contaminants, notably C4, could be removed by precipitation of a C9-rich fraction from whole serum with polyethylene glycol prior to chromatography on the Red A matrix.

Significance to Biomedical Research and the Program of the Institute: The study of the attack mechanism of the terminal C components allows a molecular analysis of a lytic pathway of the immune system. The elucidation of the biochemical events leading to specific cell destruction may provide rationale for modifying the susceptibility of foreign cells to humoral immune attack with beneficial results to the host.

Proposed Course of Project: Due to departure of key personnel, this project will be terminated.

Publications

Boyle, M.D.P. and Borsos, T.: Tissue damage caused by the direct and indirect action of complement. In Friedman, H., Reichard, S. and Escobar, M. (Eds.): Reticuloendothelial Society Treatise on Immunopathology. New York, Plenum Press, in press.

DeLisi, C., Boyle, M.D.P. and Borsos, T.: Analysis of the colloid osmotic step of complement mediated immune hemolysis. J. Immunol. 125: 2055-2062, 1980.

DeLisi, C., Boyle, M.D.P. and Borsos, T.: Mathematical analysis of the reaction of EAC1-8 with C9: Identification of parameters defining conditions for molecular titration. J. Immunol. 125: 2334-2338, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08556-05 LIB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Ultrastructural Studies of Nucleated Cells Following Modification by Physical or Chemical Means

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S. Ohanian Research Microbiologist LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Immunobiology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08557-03 LIB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunoassay of Fluid-Phase and Cell-Bound Antibodies and Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. J. Langone	Expert	LIB NCI
OTHER:	T. Borsos	Chief, Humoral Immunity Section	LIB NCI
	Y. Kato	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Immunobiology

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Humoral Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is the development and application of immunoassays for the quantitative determination of antibodies, antigens and haptens in physiological fluids or bound to the cell surface. Emphasis is placed on analysis of tumor-associated products and chemotherapeutic drugs. These assays also are used to study the interaction between antibodies and antigens and components of the complement system.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To develop sensitive and specific immunological assays which can be used to study the metabolism, disposition, and immune reactions of molecules of interest in cancer research and treatment.

Methods Employed: Immunological assays for fluid-phase or cell-bound antibodies and antigens are based on a general method developed in this laboratory in which ^{125}I-labeled protein A (^{125}I PA) (from Staphylococcus aureus) is used as a tracer for IgG antibody directed against the target molecule. Radioimmunoassay (RIA) and immunoradiometric assay, which utilize radiolabeled antigen or antibody, respectively, also are used. Other immunological and physicochemical methods including gel filtration, ion exchange and high performance liquid chromatography, thin layer chromatography, affinity chromatography (including immunoadsorption), complement fixation, electrophoresis and ultracentrifugation are used to prepare, purify, characterize, and quantify immunological and chemical products.

Major Findings: The mechanism of interaction between IgG and protein A has been studied using a competitive inhibition assay. Rabbit antibodies to human serum albumin were purified by affinity chromatography and different molar ratios of antigen to antibody were prepared by mixing equal amounts of antibody with differing amounts of albumin. The concentration of IgG in each initial preparation of immune complex was the same. Serial dilutions of each mixture were tested for their ability to inhibit the binding of ^{125}I-protein A to immobilized normal rabbit IgG under optimal conditions. Also the same molar ratios of antigen to antibody were used to obtain the precipitin curve for the ablumin - anti-albumin reaction. However, for quantification, a constant amount of ^{125}I-albumin was mixed with differing amounts of IgG. Compared to IgG alone or complexes under conditions of antibody or antigen excess, the IgG involved in complexes prepared at equivalence inhibited ^{125}I-protein A binding most effectively. These results suggest that immune aggregation and not antigen-induced conformational change is responsible for enhanced reactivity of rabbit antibodies with protein A.

Similar inhibition experiments were carried out with affinity purified rabbit IgG antibodies to methotrexate. Since methotrexate is a monovalent hapten, antibody aggregation similar to that observed with a polyvalent antigen like albumin is not possible and precipitating antigen-antibody complexes are not formed. As in the ablumin - anti-albumin system, complexes were prepared at molar ratios of methotrexate to antibody corresponding to equivalence (1/1) and large hapten excess (1000/1).

Based on the inhibition test, both sets of complexes and anti-methotrexate alone competed equally well with immobilized IgG for ^{125}I protein A. These results are consistent with the results obtained with the albumin - anti-albumin system and suggest that hapten induced conformational changes in the Fc region are not important for the enhanced reactivity between immune complexes and protein A.

In related experiments, we have used anti-methotrexate antibodies and solid supports containing different densities of hapten to show that at least two different complexes are found between protein A and immune complexed IgG on the bead surface. These complexes have been distinguished on the basis of differing affinities between protein A and IgG. The high affinity binding appears to reflect association of protein A to aggregates of IgG while the lower affinity binding involves a single protein A molecule per IgG.

In other studies we have determined that succinylated and acetylated concanavalin A, but not the native lectin, lyse sheep erythrocytes in the presence of guinea pig complement. The effect appears to be specific since succinylated wheat germ agglutinin is inactive and hemolysis is inhibited selectively by α-D-methylglucopyranoside. Hemolytic activity is enhanced by preincubation of succinylated lectin-sensitized cells with complement at 4° and correlated with the number of molecules of the first component of complement bound to cells that were treated with succinylated concanavalin A and then washed.

Significance to Biomedical Research and the Program of the Institute: Quantitative determination of antibodies and antigens is basic to the understanding of their role in immunological defense against cancer. Similarly, quantitation of bio-active agents, either endogenous compounds or chemotherapeutic drugs and their metabolic products, is fundamental to our understanding of how these agents work and may lead to a more rational basis for their clifnical use. Sensitive and specific analytical methods should help achieve these goals.

Proposed Course of Project: Improved immunological and related physical and chemical methods for the quantitative determination of tumor-associated products, cancer chemotherapeutic agents and other compounds of interest in cancer research will be developed and used to study the mechanism of antibody-antigen reactions and antibody-complement-mediated processes.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08575-09 LIB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)
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4.0	3.0	1.0

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to study the cells that participate in the effector arm of the immune response. The current emphasis is on chemotaxis, which is a mechanism by which cells can be attracted to inflammatory sites, delayed hypersensitivity reactions and growing tumors. The project includes chemistry of lymphocyte derived chemotactic factors, identification of substances that modulate chemotactic and phagocytic responses, definition and separation of functional subpopulations of leukocytes, alterations of leukocyte chemotaxis in cancer patients.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To develop quantitative measures of reactions occurring in the effector limb of the immune response; to study chemotaxis and phagocytosis of inflammatory cells in relation to this objective.

Methods Employed: Complement-derived and lymphocyte-derived chemotactic factors were generated. Human peripheral blood leukocytes were separated on Ficoll-Hypaque gradients to obtain monocyte- and basophil-rich fractions. Dextran sedimentation was used to obtain peripheral blood neutrophils. Peritoneal macrophages were harvested from normal and BCG-infected mice. Protein fractionation methods were employed to characterize chemotactic factors and serum factors that modulate mouse macrophage chemotactic responses.

Major Findings: In a quantitative study of chemotactic responses of blood monocytes from normal human subjects, we found that only a fraction of the total monocytes migrated at optimal attractant concentration and incubation time. The size of this responsive subpopulation was between 25 and 40% of the total, depending on the donor, and was not affected by various alterations of *in vitro* conditions. In particular, when the input monocyte number was changed over a hundred-fold range, the percentage of migrating monocytes remained constant. Furthermore, when monocytes were added to chemotaxis chambers, they responded to attractant without a lag, time course curves being convex to the time axis with migrated monocytes increasing monotonically until a plateau was reached. Thus the migrating subpopulation of human monocytes was responsive to chemoattractant at the moment of addition to the chamber and the response appeared to be a simple interaction of individual cells with chemoattractant, leading to directional movement. A similar analysis of mouse resident peritoneal macrophages revealed a remarkably different picture in two respects: the size of the responsive population increased with time, and the percentage of input macrophages that migrated toward attractant increased as the number of cells added to the chamber was increased. The time lag before significant chemotaxis responses occurred was shown by harvesting chemotaxis chambers at different times. A typical result with macrophage input numbers of 60,000 per well was migration of only 1000 cells during the first hour, whereas 24,000 migrated between 2 and 3 hours. Effects of cell concentration were shown in experiments with optimal chemotaxin concentrations and incubation times: only 3% of the macrophages migrated when the input number was 15,000 per well, whereas an average of 30% migrated at an input number of 120,000. Thus, responsiveness of mouse macrophages appears to be affected by cell-cell interaction on the chemotaxis membrane surface, and at the higher cell concentrations there is extensive cell-cell contact. The resident peritoneal cell population comprises lymphocytes, macrophages and mast cells. Lymphocytes and mast cells were separated from macrophages by filtration on Sephadex G-10 columns. Addition of the lymphocyte-mast cell population to fixed numbers of unseparated peritoneal cells caused an increase in the percentage of macrophages responding to chemoattractants.

As noted above, only 20-40% of human blood monocytes migrate to chemoattractants and that each of these migrating cells can respond to several different attractants. Many experimental approaches were used to determine if the 60-80% non-migrating population was due to an artifact of the *in vitro* system, and none was found. The validity of the approach was further susbstantiated by studies on

chemotaxis of cultured mouse bone marrow cells. Chemotactic activity reached a peak between days 11 and 14 of culture, with 70 to 100% of the input cells migrating. In addition to the intrinsic biological interest, the result shows that low chemotactic responses of other cells are not due to limitations of the measuring system.

Comparison of the characteristics of migrating and non-migrating leukocyte populations was made possible by the design of a separation chamber, a modification of the 48-well chemotaxis chamber. There are 4 large cell input compartments in the separation chamber, so that after the chemotaxis incubation the non-migrated cells can be aspirated from the top of the chamber and the migrated cells can be washed off the attractant side of the chemotaxis membrane. A by-product of this approach is that it provides the only available method for obtaining pure, functionally active monocytes. The purpose of the study was to compare chemoattractant binding to migrating and non-migrating populations of human monocytes. Cells were incubated with the radioactive chemotactic peptide, F-met-leu (^{3}H) phe. Binding to the migrated population was saturable at room temperature, and 50% of maximal binding occurred at $10^{-8}M$, the concentration that induced optimal chemotaxis. Non-migrating monocytes did not bind peptide under the same conditions, showing that at least one reason for non-responsiveness to chemotaxin is apparent lack of receptors. It was shown previously that exposure to superoptimal levels of chemoattractant caused subsequent unresponsiveness to that attractant without impairment of response to a different attractant (specific deactivation). Chemotactic deactivation of human monocytes with $5x10^{-7}M$ F-met-leu-phe reduced the number of available binding sites by 60%. Detection of receptor appearance and disappearance may provide a tool to investigate monocyte maturation and biochemical mechanisms of chemotaxis. For example, does absence of peptide receptors on the the non-migrating human monocytes reflect a maturational stage of the cells? The question can now be approached experimentally. In this context, the results of similar studies on human neutrophils are of great interest. The non-migrating neutrophil population had the same binding affinity and receptor number for F-met-leu-phe as the migrating population. Thus, the deficiency in the human neutrophil non-migrating population appears to be subsequent to receptor-ligand binding.

Significance to Biomedical Research and the Program of the Institute: The finding that different serum proteins affect different subpopulations of macrophages suggests that there may be regulatory serum factors that modulate macrophage motility and other functions. Analysis of the chemotaxis abnormality in patients with cancer may throw light on an early indication of the interaction between tumor and host.

Proposed Course of Project: Further characterization of the serum factors affecting function of macrophages and neutrophils. Characterization of monocyte and neutrophil subpopulations. Analysis of the chemotaxis abnormality in patients with cancer.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08576-09 LIB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunological Mechanisms of Tumor Rejection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	S. Tomisawa	Guest Worker	LIB	NCI
	J. Lazdins	IPA Investigator	LIB	NCI
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COOPERATING UNITS (if any)

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4.0	4.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is on the interaction of tumor cells and host defense cells. Current studies are on mechanisms of macrophage activation and the impairment of activation in certain strains of mice.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To determine what immunological mechanisms occur in tumor cell killing in order to provide a rational basis for elimination of tumor cells by immunological means.

Methods Employed: Chemically-induced murine tumors, maintained in tissue culture or by passage in syngeneic hosts were used. Effector immune responses were measured by cytotoxicity assays based on the release of radioactive label from damaged target cells. Protein isolation techniques were used for purification of macrophage activation factors.

Major Findings: Activated macrophages, cells with nonspecific tumoricidal and microbicidal activities, are recovered from animals with certain chronic infections such as Mycobacterium bovis, strain BCG for months after inoculation. However, regulatory mechanisms for induction and control of macrophage activation are operative for only a relatively brief interval, hours. Populations of activated macrophages that develop at sites of chronic infection or other chronic immune responses persist only because of continued presence of initiating antigens and constant replacement of lymphocyte-derived immune stimuli and responsive mononuclear phagocytes. Development of nonspecific effector function by activated macrophages during immune responses occurs after a series of reactions, each of which requires the simultaneous presence of an effective lymphokine signal and a macrophage intermediate responsive to that signal. The requirement for a reaction cascade, similar to blood coagulation or complement-mediated hemolysis, forms the basis of control mechanisms for regulation of macrophage effector function.

We have analyzed control of macrophage nonspecific effector function by several approaches:

(1.) Macrophages from certain strains of mice fail to develop nonspecific tumoricidal activity after any of several in vivo or in vitro treatments over a wide range of experimental conditions. Peritoneal macrophages from BCG-infected C3H/HeJ or A/J mice do not lyse any of a variety of tumor target cells; equal numbers of cells from BCG-infected C3H/HeN mice were strongly cytotoxic. Although the tumoricidal defects of C3H/HeJ and A/J cells appear phenotypically similar, each is mediated by distinct and genetically different control mechanisms.

Macrophages from BCG-infected but not from untreated or irritant-treated C3H/HeJ mice bind a variety of tumor cells as well as BCG-activated macrophages from the normal C3H/HeN strain. In contrast to C3H/HeN cells, however, macrophages from BCG-infected C3H/HeJ mice fail to secrete at least two activities that are directly cytotoxic to tumor cells: tumor necrosis factor and a neutral serine protease, cytolytic factor. Secretion of these soluble cytotoxic factors by macrophages from BCG-infected A/J mice was entirely normal. However, macrophages from this defective mouse strain exhibited a 75% decrease in ability to bind tumor cells. Thus, deficits in tumoricidial activity by cells from BCG-infected C3H/ HeJ or A/J mice result from mechanistically different defects in macrophage-tumor cell interaction.

(2.) Regulation of nonspecific macrophage effector function is mediated by several different lymphokines. For example, lymphokine activity for induction of macrophage tumoricidal function elutes from Sephadex G-100 as a single peak in the 50,000 mw region; lymphokine activities for induction of microbicidal effects against Rickettsia tsutsugamushi or Leishmania tropica elute in three different regions: 140,000, 50,000 and <10,000 daltons. Despite the apparent homogeneity of lymphokine activity for induction of macrophage tumoricidal effects, at least two functionally different signals can be identified. Macrophages respond to one mediator (priming signal) to enter into a noncytotoxic but highly receptive state in which they can then respond to a second lymphokine stimulus (trigger signal) to develop tumoricidal activity. Priming signals are effective at very low concentrations (1/500 dilution of active leukocyte culture supernatant) and and require 4 hours for optimal response; trigger signals are effective only at high concentrations (1/20) of the same supernatant but require less than 10 minutes for optimal response. It is the trigger signal that is limiting during immune reactions. Lymphokine priming and trigger signals form the basis of a regulatory system that sets the threshold and determines the onset of macrophage effector function.

(3.) Development of tumoricidal activity by macrophages treated in vitro with lymphokines follows a reproducible time course: cytotoxic activity is evident by 2 to 3 hours, becomes maximal by 6 to 12 hours and then progressively decreases to control levels by 24 hours. Loss of tumoricidal activity with time is not due to cell death or exhaustion of lymphokine. There are several major alterations in cell lipid content that correlate with the time for onset and loss of tumoricidal activity. These lipid alterations were evident 2 hours after lymphokine treatment; maximal changes occurred by 4 to 6 hours and progressively returned to initial values by 16 to 24 hours. At the maximum, total cell lipid increased 3 to 5 fold; cholesterol and triglyceride levels increased 5 to 10 fold. Saturated fatty acids in untreated macrophages comprised about 90% of the total fatty acid. In contrast, 75% of the fatty acid content of lymphokine-activated macrophages was unsaturated.

Activated macrophages from mice treated with viable BCG or killed Corynebacterium parvum show changes in lipid and fatty acid content similar to lymphokine-treated cells. Moreover, macrophages from C3H/HeJ mice respond to lymphokines by the criteria of increased glucose consumption but do not become tumoricidal; lymphokine-treated C3H/HeJ macrophages fail to develop the characteristic changes in lipid or fatty acid composition. Thus, macrophage activation for nonspecific tumoricidal activity in vivo and in vitro is associated with alterations in cell lipid content.

Significance to Biomedical Research and the Program of the Institute: Analysis of the effector immune events leading to tumor rejection may provide a rational basis for manipulation of host responses or tumor cell in an effort to eliminate or prevent progression of the tumor.

Proposed Course of Project: Studies on the mechanisms of the killing of tumor cells by macrophages.

Publications

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Annual Report of the Laboratory of Cell Biology

National Cancer Institute

October 1, 1980 - September 30, 1981

The following are selected highlights of the research efforts of the Laboratory of Cell Biology.

I. Histocompatibility Antigens

a) Purification and properties of H-2^b

The purification of H-2^b and a preliminary characterization of artificial liposomes was already reported. In the earlier studies it appeared that some H-2^b was completely resistant to papain digestion. We have now determined that this resistance was due to some H-2^b being bound to multilamellar liposomes and thus buried in lipid and protected from proteolysis. When liposomes are prepared under conditions where mainly unilamellar liposomes are produced, only two forms of H-2^b were observed, both susceptible to papain digestion. About half of the H-2^b is oriented toward the outside of the liposome and is cleaved by papain to produce a 44,000 dalton fragment which is released from the liposome and contains all of the alloantigenic sites. The other half is oriented toward the inside of the liposomes and is cleaved by papain to produce a 47,000 dalton fragment that remains bound to the liposome. No intact 50,000 dalton H-2^b remains.

Liposomes containing H-2^b have now been successfully used to stimulate primed, allostimulated spleen cells to produce cytotoxic T cells. Spleen cells from B10A (H-2^a) animals primed with B10 (H-2^b) spleen cells were incubated for 5 days with liposomes containing H-2^b. After 5 days cytotoxic T cells were isolated from these incubations which could kill RBL-5 (H-2^b) tumor cells but were unable to kill YAC (H-2^a) tumor cells. The effects of 1) varying the density of H-2^b on the liposomes 2) varying the concentration of liposomes and 3) removal of adherent cells before secondary stimulation are under investigation.

In collaboration with Dr. Linda Gooding we are planning to expand these artificial liposome experiments to include another immunogenic protein incorporated into liposomes along with H-2^b. Human erythrocyte glycophorin has been chosen since it is immunogenic, has been shown to bind to artificial membranes and can be easily purified. Our lab at NIH will concentrate on defining the effects of glycophorin on the immunogenicity of H-2^b in the <u>in vitro</u> secondary stimulation experiments described above. Dr. Gooding will concentrate on the immunogenicity of glycophorin on these liposomes. Initially, liposomes containing glycophorin will be fused with spleen cells and these will be used as targets to search for cytotoxic glycophorin specific T cells in the spleens of animals immunized with either

H-2 + glycophorin liposomes or glycophorin containing spleen cells.

b) cDNA clones encoding mouse histocompatibility antigens

We have obtained cDNA libraries from poly(A)$^+$RNA isolated from ascites cells of LSTRA and screened the libraries with a cDNA clone encoding a human transplantation antigen. Three cDNA clones were identified. One clone was sequenced and found to encode amino acids 50-287 of an H-2 like antigen with 90% homology to the published protein sequence of H-2K^b. Comparison of this cDNA sequence with both β_2-microglobulin (β_2M) and the constant region domains of mouse μ immunoglobulin gene revealed a striking homology which suggested that the three genes shared a common ancestor. Restricted enzyme digests of liver DNAs from different inbred strains of mice were hybridized to such probes and 10-15 bands were found for each strain; these results indicate that the histocompatibility antigens appear to constitute a multigene family.

c) Control of the expression of mouse histocompatibility antigens and β_2M in F9 teratocarcinoma stem cells

F9 teratocarcinoma stem cells differ from most other cell types; these cells do not express any major histocompatibility antigens. Evidence has been obtained that this regulation of major histocompatibility antigen expression is due to transcriptional control of the major histocompatibility antigen genes (H-2 and β_2 microglobulin). The steady state levels of β_2 microglobulin and H-2 mRNA from F9 teratocarcinoma stem cells and differentiated cells were examined by "Northern Blot" hybridization using cloned DNA probes specific for these mRNAs. F9 teratocarcinoma stem cells (clone 12-1) contain at least ten fold less H-2 and β_2 microglobulin mRNA than the differentiated daughter cells (clone 12-1a). The transcriptional regulation of these genes is accompanied by a change in their DNase I sensitivity. Normally transcriptionally inactive genes are DNase I resistant, while active genes are DNase I sensitive. In contrast, the silent major histocompatibility antigens of teratocarcinoma stem cells are more DNase I sensitive than the active genes of the differentiated cells. Commitment to H-2 or β_2M expression apparently is not correlated with a chromatin conformation which confers increased sensitivity to DNase I digestion as has been observed in other systems such as globin, adenovirus and ovalbumin genes.

d) Control of expression of H-2 antigens on mouse placenta cells

Because of the placenta's capacity for rapid cell replication, this organ is an excellent model for the study of normal and abnormal cell replication and for investigating the regulation of gene expression.

In our first attempt to use such a system, we have utilized two established cell lines of BALB/c and C57BL/6 origin which appear to be trophoblasts as judged by their production of gonadotropin-like substance and steroid hormones. These cell lines do not express H-2 and β_2M on the membrane. They synthesize only very low amounts of the two proteins. Analysis of the mRNAs present by hybridization to specific probes revealed that substantial amounts of the RNAs are present. The defect in expressing of H-2 and β_2M is therefore at the translation level. Further work is being carried out to assess the molecular events which are involved in the regulation of the expression of these genes.

e) Purification and characterization of mouse β_2 microglobulin variants from two different strains

Variants of β_2M (β_2m^a (BALB/c strain) and β_2m^b (C57BL/6 strain) first detected by Michaelson et al. (Immunogenetics 11: 93-95, 1980) have been purified to homogeneity in reproducible yields (10-12%) using an improved method. The purifications were accomplished by a 3M sodium thiocyanate extraction of a crude membrane fraction, an acid precipitation step, gel filtration on Sephadex G-75 and ion exchange chromatographies on DEAE-cellulose and CM-cellulose respectively. Charge differences observed between the two variants on ion-exchange columns were consistent with differences in pI values and in amino acid composition. β_2m^a was found to be more acidic (pI = 7.35) than β_2m^b (pI = 7.65). β_2m^a contained 10 aspartic acids and 3 alanine residues while $\beta_m{}^b$ contained 9 aspartic acid and 4 alanine residues; no other differences in the amino acid composition were observed. Structural evidence for the strain related polymorphism of β_2M was obtained by a) determination of the primary structure of β_2M and b) partial sequence analysis of β_2m^b. Complete sequence analysis of β_2m^a was performed by an automated Edman degradation of the intact chain and of the overlapping peptides obtained by (a) tryptic cleavage at arginines after acetimidation of lysines; b) BNPS-skatole cleavage at tryptophan and c) hydroxylamine cleavage at asparagine-glycine linkages. A comparison of the primary structure of β_2m^a to the partial sequence obtained for β_2m^b revealed that a single amino acid substitution (aspartic acid to alanine at position 85) accounts for the biochemical differences observed.

f) Mouse β_2M cDNA clones

We have isolated three cDNA clones for β_2 microglobulin. β_2 microglobulin comprises less than than 0.1% of mouse liver protein, and its mRNA is approximately 0.03% of liver poly(A)$^+$ mRNA. The cDNA clones were identified by screening 1400 cDNA clones made from 9-10S mouse liver poly(A)$^+$ mRNA. The procedure for screening the cDNA clones involved binding pooled plasmid DNA to nitrocellulose filters and testng the ability of each filter to select β_2M mRNA. The filter selected mRNAs were assayed for their ability to direct the synthesis of β_2M in in vitro translation reactions. The

isolated clones were shown by nucleotide sequence analysis to encode β_2M. The positive-selection hybridization assay has been modified to facilitate the screening of large numbers of cDNA clones, and should allow the isolation of cDNAs corresponding to any mRNA whose in vitro translation products can be immunoprecipitated. The cDNA clones for β_2M contain sequences corresponding to the entire β_2M mRNA with the exception of the 5' and part of the 3' untranslated regions and the regions encoding the signal peptide, amino acids 1-10 and residues 42-49. These clones provide nucleic acid probes to examine the structure of the β_2M gene(s). Preliminary characterization of mouse β_2M gene sequences by Southern blot analysis suggests that there is only a single complete β_2M gene. This gene has two intervening sequences located near the ends of the coding sequences.

g) Isolation of SLA antigens

We have prepared and purified papain fragments of the major translation antigens (SLA) from two of three herds of miniature swine. The SLA heavy chains have been separated from non-covalently associated β_2-microglobulin and NH_2-terminal sequences determined. We have identified the N-terminal 40 amino acids from one haplotype and 36 from the second, as well as 45 from the associated β_2 microglobulin. With the exception of the human, these are the longest transplantation antigen sequences so far determined using non-radioactive (micro-sequencing) techniques. Appropriate comparisons have been made with available sequences from other species.

Since the amount of material required for a complete protein structure would be considerable, we have chosen to use purified antigens for biological experiments and to determine the structure of these proteins through analysis of their corresponding genes. Using a cDNA probe to human transplantation antigens we have been able to characterize porcine SLA and SLA-like genes by 'Southern' blot analysis. We have also isolated a genomic clone containing SLA sequences by screening of a genomic phage library with the human probe. Experiments are now beginning to characterize and sequence this clone. Using this same procedure we have similarly isolated a genomic mouse H-2 clone which will eventually be used to study the evolution of H-2 genes in our wild mouse colony.

In addition to the transplantation antigens the major histocompatibility complex also encodes a series of gene products involved in the regulation of the immune response and cell-cell interaction. These I region gene products have been characterized in our three herds by two-dimensional gel analysis and attempts to purity these molecules are currently underway.

II. Tumor antigens

a) The soluble tumor antigens of BALB/c sarcoma Meth A

The cytosol of the BALB/c sarcoma Meth A contains both a serologically defined tumor specific surface antigen (TSSA) and a tumor associated transplantation antigen (TATA). The relationshp between these two antigens has been investigated. Chromatographic fractions derived from the cytosol of Meth A cells which possess a soluble TATA were assayed for the serologically defined TSSA by absorption analysis. Meth A cells were lysed and a high speed supernatant (HSS) prepared. This HSS was treated with 1.25% streptomycin sulfate and nucleic acids were removed by centrifugation. The supernatant was brought to 55% $(NH_4)_2SO_4$ saturation and the protein pellet dialyzed overnight. The dialysate was then applied to a hexylamine-agarose column and eluted with a gradient of 0-1.0 M NaCl. Four main fractions were eluted from the column. The majority of the TATA activity (80%) was recovered in the unabsorbed fraction (MHo). The MHo fraction was found to be specific in the in vivo tumor rejection assay. The MHo fraction was further chromatographed on an Ultrogel AcA44 column. Five fractions (F_I-F_V) were recovered which covered a range of molecular weights between 170K and 10K. The high molecular weight fraction, F_I (~170K) contained 80% of the original activity while F_{II} (~67K) contained about 10% of the activity. The first four fractions (F_I-F_{IV}) were assayed for the serologically definedTSSA by absorption analyses; only F_I which was enriched 25 fold in TATA showed complete absorption of cytotoxicity. Furthermore the absorption was specific only for the Meth A antiserum and not for a similarly prepared antiserum defining a TSSA on the CMS4 tumor. The AcA44-F_I fraction was chromatographed on hydroxylapatite to give five fractions HA-1A, 1B, 2, 3, and 4. All HA fractions were tested in the in vivo tumor rejection assay. Only HA-2 (major component 65K, pI 5.8) and HA-4 (major components 72K and 76K) gave substantial inhibition of tumor growth. When these same fractions were assayed by absorption analysis, only HA-2 showed partial absorption of cytotoxicity. The AcA44-F_I fraction was also subjected to preparative SDS polyacrylamide gel electrophoresis. This technique yielded highly purified fractions of the 65K, pI 5.8 component and the 76K pI 6.3 component. At doses of 10 µg in carrier BALB/c serum, the 65K and 76K proteins both provided complete protection against Meth A tumor growth. Thus our studies to date indicate that there are two proteins possessing TATA activity. The 65K component is extremely sensitive to proteolysis and this fact suggests that it may be a lower molecular weight form of the 76K protein. The exact relationship between these two proteins is currently under study. The purification and characterization of a discrete TATA now allows the exciting exploration of the relationship between the genetic determinants of transformation and a unique TATA of Meth A sarcoma.

b) Antigens of CI-4 sarcoma

Another approach to purification of TATA is being used. Meth A and CI-4, another MCA-induced BALB/c sarcoma of recent origin, will both be purified simultaneously from the cytosol using high salt and dithiothreitol to prevent aggregation. Purification has proceeded through extraction and gel filtration and we find that CI-4 behaves like Meth A in that: the soluble (not solubilized) cytosol contains most of the TSTA activity which is specific for CI-4 and behaves similarly to the membrane-derived TSTA. Small amounts of material (~10μg) give complete protection against CI-4 challenge. Antisera have been obtained from rabbits. It is the hope that the impurities present at this stage will be common to Meth A and CI-4 and that absorption of the impurities against CI-4 may be used to absorb the impurities against Meth A (and vice versa) leaving TSTA as the unique protein.

The immunogenicity and therapeutic efficiency of soluble partially purified CI-4 TATA bound to artificial membranes (egg lecithin liposomes) have been studied. Preliminary results reveal 1) the liposome-bound CI-4 TATA is more effective than CI-4 alone and 2) the CI-4 liposome material is very effective if given as long as 5 days in 5 μg-50 μg amounts after challenge with CI-4 (therapy regime).

c) Attempts to isolate and further characterize the 11A antigen

We continue our studies of the mechanisms involved in the specific Meth A immunogenicity found in the cells of an M-MSV (MuLV)-induced BALB/c sarcoma. It was found originally that a tissue-culture passaged variant of the neoplasm, 11A, was capable of absorbing cytotoxicity of the specific α-Meth A antiserum, the only one (except Meth A) of more than 100 neoplasms, normal tissues and viruses capable of absorption. The 11A variant was found to immunize BALB/c mice in a specific manner against Meth A only. The 11A variant is a virus producer line but in contrast to the original 11A tumor line (carried in vivo) does not contain rescuable MSV.

An endogenous B-tropic retrovirus has been isolated from the 11A variant line. This uncloned virus (11A-MuLV) when infecting SC-1 cells was found to transmit specific immunity to Meth A. 11A-MuLV was cloned by twice limiting dilutions but upon infectivity of SC-1 cells was negative in specific immunogenicity assays against Meth A suggesting that 11A-MuLV represents a minority among the 11A-MuLV population of viruses. Then, SC-1 cells were infected with a higher multiplicity of virus (sucrose-banded virus). The infected SC-1 cells were cloned and assayed for XC and RT reactivities and for their ability to absorb cytotoxic αMeth A antibody. Among the many clones assayed and those showing XC (+) reactivity, about 20% (9 clones) were positive for absorption. These (+) clones assayed for Meth A immunogenicity and only 2 clones (#s 36, 46) were immunogenic against Meth A. Recently however, 2 11A-infected clones (#s 17 and 22) were found to be SC-, RT+ and absorbed Meth A cytotoxicity. These clones produced virus particles and immunized against Meth A - but only in the early passage

(not late passage) generations. If virus and TSTA are coordinately expressed, these results suggest that virus replication is inefficient and that successive cell passages reduce the number of progeny virus. These observations are consistent with other data indicating defective virus. Nonetheless early passages of virus in SC-1 cells may provide the source for isolation of the 11A defective virus.

d) p53, a transformation associated antigen

Malignant cells of the mouse or other species, whether transformed by viruses, chemicals or x-ray, express a cellular protein with a subunit molecular weight of 53,000. Detectable levels of p53 were not found in a broad range of normal cell types. A 53,000 molecular weight protein has been detected in transformed B cells of human origin but not in non transformed B cells. In cell lines carrying the Epstein-Barr virus-directed EBNA antigen, the p53 component forms a complex with EBNA. The nature of the regulatory defect that leads to persistent high levels of p53 in transformed cells remains an important question. In order to answer this question, we have attempted purification of the protein in order to characterize its structure and construct nucleic acid probes that detect the p53 gene. Our preliminary results (Jornvall, Luka, Klein and Appella) indicate that purified p53 obtained from mouse or human transformed cell lines have very close amino acid compositions and sequences of the first 20 residues. Further work now in progress will be directed toward the elucidation of the complete amino acid sequence in order to understand the function of this protein and its role in the regulation of cell division.

III. Transfection with tumor DNA

Our collaborative study with the Sloan-Kettering group (Drs. DeLeo and Old) and with Dr. Nancy Hopkins (MIT Cancer Center), concerning the phenotypic expression of Meth A specific tumor antigens (TSTA and TSSA) continues and has yielded interesting results. BALB/c 3T3 (f7) subline, specifically cloned and selected has been transfected with high molecular weight DNA from Meth A sarcoma and appropriate controls. The following assays done blindly have been accomplished from transformed foci:

1) assay for malignancy

2) presence of TSSA by absorption of the specific cytotoxicity of a Meth A syngeneic antiserum, and

3) tumor rejection and specificity

We have observed a high frequency of co-transfection of transformation and TSTA. All transfected lines (from Meth A DNA) grew progressively in BALB/c mice; 5 of 8 foci selected from one experiment possessed Meth A TSTA. DNA extracted from one of these lines was used in secondary rounds of transfection; 4 of 5 of II transfectants contained Meth A-specific TSTA. These results suggest that in the

Meth A sarcoma a transforming gene and a genetic determinant of TSTA are intimately related since one would expect the frequency of a transfer of the 2 phenotypes to be only 0.01 to 0.001 if specified by unlinked genes.

Some of the transfected clones also absorbed cytotoxicity of the Meth A antiserum but this assay appears less sensitive than the in vivo TSTA assay.

One of the transfected lines (BMA-11-7a) has been passaged continuously in vivo and assayed through 15 generations for immunizing capacity against itself and against Meth A and for immunosensitivity. Results show both phenotypes to be stable.

Some of the Meth A DNA cleaved with restriction enzymes retain transforming capacity (Hopkins and Vande Woude); these are now being assayed for the presence of Meth A TSTA. This DNA fragment appears to be of a size (15 Kb) appropriate for molecular cloning. A report of this work has been submitted for publication (Hopkins, Besmer, DeLeo and Law "High frequency co-transfer of the transformed phenotype and a TSTA using DNA from the MC-induced Meth A sarcoma of BALB/c mice").

This investigation is continuing in the following aspects: 1) assays for conversion to malignancy in the transformed foci (transformed in tissue culture) and assays for the TSTA phenotype in the "cut DNA" series, 2) use of another MC-induced sarcoma (CI-4 or CII-10) for transfection studies to determine the generality of these transfections.

A real possibility exists therefore of an assay that will allow the transfecting genes to be cloned with resultant understanding of the chemical carcinogenesis mechanisms.

IV. Immunoglobulin structure, genetics and diversity

a) Karyotypic analysis of plasmacytomas

In collaboration with Dr. G. Klein of the Karolinska Institute, a karyotypic analysis of primary and early transfer generation plasmacytomas has been carried out using G-banding to identify specific chromosomes. Among 18 tumors analyzed several characteristic translocations have been identified: T6;15, T12;15, deleted 15. Sixteen of the 18 tumors have the T12;15 and del 15 abnormality and 3 have the rcpT6;15. All of the latter are kappa chain producers. In a series of 8 lambda tumors none has rcpT6;15. Since chr. 6 carries the K-chain genes and chr. 12 carries the heavy chain genes, there seems to be some association with Ig-gene complex loci. This is further strengthened by the observation that the translocations involve only 1 haplotype in a cell and in tetraploid cells only 2 of the 4 chromosomes are involved. The break in chr. 15, however, is consistent throughout and appears to occur in the same band site. Because trisomy of chr. 15 is implicated in T-cell leukemias in the mouse, it is possible that a break in chromosome 15 may have an

important biological effect on the differentiation and development of lymphocytes.

We are also attempting to induce plasmacytomas in mice carrying the T6 translocation. T6 is carried on a CBA background a mouse that is presumably plasmacytoma resistant. The T6 translocation involves chrs. 14 and 15. Thus, if plasmacytomas could be induced in mice that have a normal 15 and a 14;15 translocation, it would be of interest to determine which chromosome develops the T12;15-del 15 translocation. For this purpose we are introgressively backcrossing T14;15 and del 15 (from T6) onto BALB/c and attempting to induce plasmacytomas with pristane. We have also obtained two other translocation stocks for this purpose T10;12 31H, T5;12. These translocations are also on plasmacytoma resistant strains.

Since little is known about chr. 15 in the mouse we are studying the effects of other marker genes and their neighbors located on chr. 15 for their role in plasmacytomagenesis. We are testing bt (belt) on C57BL/Ka which can be assayed at BC_2; caracul (Ca) which probably can also be similarly assayed. The presumption here is that genes controlling susceptibility in BALB/c are located on chr. 15.

b) Susceptibility to plasmacytoma induction

An approach to finding genes that are associated with susceptibility and resistance is to compare the susceptibility of BALB/c sublines. We are currently submitting for publication a study of BALB/c J and BALB/c Boy. BALB/c J develop only 10-20% plasmacytomas as compared with 60% in BALB/c An and BALB/c An Boy. We are currently examining BALB/c Argonne, and will soon study BALB/c ORNL and BALB/c. The last 2 mentioned substrains were separated from BALB/c An over 46 years ago.

The partial resistance of BALB/c J provides a model system for studying the genetics of plasmacytoma susceptibility. A survey of genetic differences including over 50 genes (Rodericks' Alpha gene survey) had only 1 difference (Qa-2 and Qa-3). It is also known that BALB/c An and BALB/c J have differences in genes controlling catecholamine biosynthesis. We are attempting to find new genetic differences by using DNA probes, and to this end have begun with retroviral gene probes. Differences have been found in flanking sequences around MTV and a xenotropic type C probe. These are being pursued as possible clues. We hope to make probes specific for the flanking sequences and then to use these as genetic markers.

A working hypothesis is that BALB/c is susceptible to developing plasmacytomas because (differentiation specific) genes controlling plasma cell proliferation are regulated in a special way. This could be caused by genetic events that involve the regulatory elements of these putative genes. Candidates for such events are retroviral gene insertion, or consequences of retroviral gene recombination with host genes.

c) Galactan binding myeloma proteins

We previously described the complete amino acid sequences of the κ light chains from 6 myeloma proteins with specificity for β(1,6)galactan containing antigens. It was noted that among these proteins all four J (joining) region segments were used in conjunction with a single κ variable region and that no changes in specificity or affinity were observed in spite of these structural differences. Furthermore, position 96 at the junction of V and J was not encoded in either V or J but was generated by the recombination event and in 5/6 proteins was Ile. To assess the fidelity of this unusual V-J recombination and the diversity among anti-galactan antibodies we have begun amino acid sequence anaylsis of 11 hybridomas demonstrating the same specificity. Complete sequence analysis of these hybridomas will permit an evaluation of structural diversity, functional diversity, idiotypy and the mechanisms involved in the generation of these molecules. Correpondingly, we have isolated messenger RNA coding for both the light and heavy chains from one of the anti-galactan myelomas. We have constructed cDNA from this message and the cDNA has been cloned in a plasmid vector. Nine kappa and four alpha chain clones have been isolated and are currently being characterized. If any of these clones possess sequences corresponding to their respective V regions they will be used to isolate and characterize the germ line genes coding for these molecules. Thus, a complete description of anti-galactan antibodies from genotype to phenotype will be possible.

d) VH-isotypes

The Vk structures produced in the BALB/c mouse have a diversity of structures in the first framework region of the chain (positions 1 to 23). Amino acid sequence analysis of this segment can potentially provide data on the number of Vk-genes in the BALB/c genome. Seventeen new Vk-partial sequences have been completed by S. Rudikoff. We have sent these sequences to Drs. E. Haber and John Newell, Massachusetts General Hospital, who are analyzing them for relatedness and the construction of a dendrogram based on the number of nucleotide differences. It is anticipated that this dendrogram will provide a basis for predicting the total number of BALB/c-k genes.

e) Studies of organization and control of genetic material in immunoglobulin-secreting plasmacytomas

Structural studies of cDNA and genomic clones of BALB/c IgD heavy chain (δ chain) constant region have demonstrated that the δ gene consists of exons which encode two constant region domains, Cδ1 and Cδ3, a hinge region CδH, separating the two constant domains, and at least 4 exons which appear to encode alternate 3' termini for different δ chain proteins such as those which are membrane bound and those which are secreted. In plasmacytomas TEPC1017 and TEPC1033, which secrete IgD as well as bear IgD on the cell surface,

we have identified 4 RNAs that hybridize to the δ chain cDNA clone. The smallest of these δRNAs, containing 1750 nucleotides (NT) is the most abundant RNA and is felt to be the mRNA for secreted δ chains. The next most abundant RNA, containing 2900 NT, is felt to be the mRNA for membrane bound δ chains, because this is the most abundant δRNA in normal spleen cells. Hybridization studies of these RNAs using probes from the different genomic exons, as diagrammed below, indicate that RNA splicing attaches the DC gene segment to the secretory δ message of 1750 NT and the VVDC gene segment to the 2900 NT RNA for membrane bound δ chains. The minor bands consisting of 2100 NT and 3200 NT, contain VVDC and DC coding regions, respectively. The 3200 NT δRNA is probably the precursor of the mature 1750 NT mRNA, but the 2100 NT species remains a mystery. It could be a scarce, short-lived mature membrane δ mRNA derived from a 2900 NT precursor, or since it has not been detected in normal spleen RNAs it may be an RNA found only in tumors.

Southern blot experiments with genomic DNAs indicate that in normal spleen cells and in μ + δ containing lymphomas the μ genes and δ genes retain their germ line configuration separated by 4600 bp of DNA. However, in TEPC1017 and TEPC1033 a VDJ region complex appears to have switched to a position between these genes causing deletion of the μ genes. This rearrangement is thought to take place in those rare instances when B cells differentiate to become gD secreting cells.

V. Mammalian Cellular Genetics

a) Culture of differentiated cells.

Our Fisher Rat Thyroid cells grown in low or no serum (FRTL) have continued to provide a powerful resource; they are the first and possibly only example of a karyotypically normal, long term cell strain that has retained complex differentiated functions including biosynthesis of physiological amounts of thyroglobulin (TG), concentration of 100-fold or more of iodide, and apparently normal response to the hormone thyrotropin (TSH). We have sent these cells out to laboratories around the world and they have provided the basis for numerous studies involving hormone mechanisms - especially the response of the cyclic AMP system to TSH stimulation.

However useful these rat cells may be there is always a premium paid on having a human cell strain available. After Drs. Ambesi-Impiombato and Coon succeeded in developing strategies that were successful in getting rat cell strains they thought that surely there would be no difficulty in getting human cell strains in the same way. That was not to be. Human cells have been supplied by Dr. William Valenti from surgical procedures on diseases thyroids which necessitated removal of small amounts of apparently normal tissue as well as from the "instant autopsy" program of the University of Maryland. In collaboration with Drs. Ambesi-Impiombato, Valenti, and L. Kohn we have made many attempts to culture human thyroid cells without success. Recently, during his visit from Naples, Dr. Ambesi has

succeeded in overcoming the "human thyroid program". The same media and hormone supplements used for the rat cell lines are used but no serum can be tolerated by the human cells at any time. Furthermore, they require self conditioned medium for long term survival. It has been known for some time that certain tumor cells and transformed cells may produce growth stimulating factors in the culture medium and recent evidence suggests that even normal cells may produce growth factors such as insulin (albeit in very small quantities). Apparently the human thyroid cells require a factor that they themselves synthesize. This sort of "autocrine" phenomenon may be related to the chelones and other similar growth regulating substances that are vital to maintaining the integrity of groups of cells in tissues and may very well have important implications for the future understanding of the cancer problem. At any rate, the availability of human cell strains with thyroid function appears near reality. Such cell strains, if they are like the rat cell lines will prove important developments. Whenever we learn something about how to grow normal, differentiated cells in culture we are getting closer to understanding how these cells are integrated into tissues and organs and are therefore, closer to understanding how those mechansims can go astray.

b) Continued work with the FRTL strain

We have begun an effort to produce variants (mutants) of the FRTL cell strain that no longer produced TG. Such nonproducers are not seen in our normal cultures when they are screened with FITC-anti TG. Small clumps of TG are always seen on the surface of every FRTL cell (they are not seen on FRT (a non-functional epithelial cell from the thyroid that we believe has secondarily lost the ability to synthesize TG). We determined that the best way to screen for TG-nonproducers among mutagenized FRTLs would be to adapt the agar + medium + antiserum overlay technique developed by Coffino, Laskof and Scharff for similar screening for IgG-nonproducers. This procedure worked very well indeed. We produced rabbit anti-rat-TG and incorporated it in agarose + 6H medium and overlayed the clones grown from populations of FRTL that had been exposed to standard mutagens (e.g., EMS). After 24-48 hours a precipitate could be plainly seen over the majority of colonies and none was seen over a very few (estimated one putative non-producer per 2000 colonies). Some colonies seemed exceptionally active. Both types of colony are being isolated for further study. Using techniques like subselection and/or further mutagenesis it should be possible to produce a wide variety of mutants deficient and superficient in different portions of the system that regulates TG production. To date, the most significant part of these results is the development of a successful screening technique.

Work has continued on the electrophysiology of the FRTL cells. The ionic mechanism of thyroid cell secretion has been studied in response to catecholamines. We have recorded from single cells

and iontophoresed norepinepherine onto the external cell membrane (norepinepherine causes depolarization of the membrane potential). We have perfused medium with altered Na+, Ca++, Cl- or K+ concentration outside the cell to determine ions responsible for response to conductance to Cl- and Na+. Both Cl- and Na+ conductances are increased by release of intracellular Ca++. Injection of EGTA from a second intracellular electrode abolished depolarization to norepinepherine depolarization.

Perhaps the most exciting development of the past year was our discovery of slow oscillations in thyroid cell membrane potential. These oscillations of 10-30 mV amplitude and 5-10 min period represent a hitherto completely unknown and unobserved property of secretory cells. Because of the thyroid's strategic role in regulation of metabolism in virtually all other cells, the impression of such a cyclic clocklike influence on the body may be a profound one indeed. As yet, however, we have not linked the periodic membrane potential changes with secretion. If that is done then there will be profound medical implications as well as implications to theoretical biology of this phenomenon.

Some details that are now emerging: Oscillations occur spontaneously in some cells and are induced by injecting H+ ions in others. Also, they are recorded in greater fraction of cells in low TSH medium. We have not been able to record any change (increase or decrease) in ion conductance associated with the oscillations. Thus, it may be some electrogenic ion exchange, for instance Na+, H+ which is regulated by internal H+ concentration. This implies some physiological role, unknown but perhaps TG secretion is regulated by these oscillations. The results predict that CO_2 saturated medium would increase TG secretion. In the near future, we will try to measure the intracellular pH to see if there is a correlation with amplitude or period; also, to see if intracellular pH of low TSH grown cells is different from those grown in the usual concentration (2 mU/ml). Also, we will try to measure single Cl- ion channel conductance turned on by norepinepherine using an extracellular patch electrode attached to a 1 um diameter piece of external cell membrane. If we can do this we will then be able to determine more about the Ca++ regulation of channel open time and conductance.

VI. Miscellaneous findings

a) Effects of interferon on murine retroviruses.

Most of the studies in this system are being done with Dr. Paula Pitha and reflect a continuing interest in the molecular mechanisms which account for the effect of interferon on retroviruses. In the case of most viruses, interferon causes an impaired mRNA translation

with resulting defects in viral structural proteins. However, the effects of interferon on retroviruses occur after the synthesis of viral RNA and most of the structural protenis. It is now well established that the predominant mode of action concerns the late stages of virus assembly and maturation. Further, the precise type of change observed seems to vary among the different classes of retroviruses. Yet the end result is essentially the same, the productio of particles which are noninfectious. It must be emphasized that this interference with assembly is dependent on the continuous presence of interferon in the in vitro system; following withdrawal of interferon there is an almost complete recovery of virus titer within 18-24 hours. In the cse of ecotropic viruses such as Gross and Rauscher murine leukemia virus, there is maturational arrest at either the budding or release stage depending on the type of host cell used for propagation. These ultrastructural observations correlate with a decreased rate of proteolytic cleavage involving the high molecular weight polyprotein precursors of the env and gag gene products. As a result the noninfectious particles contain env pr85 and gag Pr65 as well as gp71 and p30.

Our most recent experiments have been done with a new class of murine retrovirus, MCF, which is apparently an env gene recombinant of ecotropic and xenotropic viruses endogenous to the AKR mouse, and which seems to accelerate the development of leukemia. One of the characteristics of MCF virus is a slow rate of processing of the pr76 env precursor to gp71, as compared to the ecotropic viruses. Since previous studies with ecotropic viruses indicated altered rates of proteolytic cleavage of the env gene products, the strategy in using MCF was to exploit an assembly stage in which the kinetics of cleavage are already slower than average. Notwithstanding this initial thesis, the results reflect a more complex situation than originally anticipated.

b) Xenotropic virus isolation and characterization

Further characterization of xenotropic viruses (isolated from SJL/J mice and Abelson tumor of BALB/c mice), a dual-tropic virus (isolated from Abelson tumor) and amphotropic viruses (isolated from RBL-5 and YAC tumors) by competitive radioimmunoassay.s

The type-specific p12 competitive assay results for SJL-MEF-X-MuLV and SJL-RCN-X-MuLV indicated that these viruses were more closely related to BALB:virus-2 than to NZB-X-MuLV or AT 124--MuLV. This result was consistent with the fact that SJL/J as well as BALB/c mice produce both ecotropic and xenotropic viruses while NZB and NIH Swiss mice produce only xenotropic viruses (NZB- and AT 124-MuLV respectively). Abelson-X-MuLV is also more related to BALB:virus-2 than to NZB-X-MuLV, but it has its own uniqueness as shown by p12

assays. The dual-tropic NBX virus isolated from Abelson tumor is a recombinant between Moloney-MuLV and X-MuLV as indicated by p12 and gp70 competitive radioimmunoassays, but it acquired a unique gp70 antigen, the origin (parent virus) of which is unknown at the present time.

Competitive radioimmunoassays with new isolates of amphotropic viruses showed that R5NX (from RBL-5 cells) has p12 which is related to AKR p12 while YACNX has no such cross-reacting antigen. This fact is in accord with the tryptic digest map reported previously Further work is being done to characterize these and other newer isolates.

c) Further studies on the interferon and natural killer (NK) activity associated with transplantable and spontaneous reticulum cell neoplasms (RCN) of SJL/J mice

Recent investigations revealed the presence of immune interferon (IFN-γ) (acid-labile) in the RCN-homogenate. Intraperitoneal injection of RCN into SJL/J mice induces a transient production of acid-stable interferon detectable in the serum of mice 1 day after injection, but the IFN-γ appears in the lymphoid organs only when the RCN has grown to a certain extent. The increase in NK activity in these RCN preparations may be accounted for by the action of IFN-γ , triggering activation of pre-NK cells to become mature NK cells as well as enhancing the killing efficiency of pre-existing NK cells. The mechanisms by which IFN-γ is induced in the RCN-carrying organs is not clear.

In contrast to transplantable RCN, the spontaneously arising, primary RCN in the spleen and lymph nodes of older SJL/J mice display generally weaker NK activity and different range of target cells. Mice bearing transplantable RCN showed a high NK activity against RBL-3, EL4G$^-$, and YAC tumor cells but no activity against a syngeneic lymphocytic tumor, D2, whereas those bearing spontaneous RCN showed a high NK activity against EL4G$^-$ and/or YAC tumor cells, low, if any, against RBL-3 cells, and often high against D2 cells. These activities could not be abolished by removal of macrophages and nylon wool-adherent cells or by treatment with anti-Thy 1.2 serum plus complement.

These obervations suggest that different subpopulations of NK (or pre-NK) cells are being activated during primary development and subsequent transplant passage, each subpoulation representing a clone of NK cells with different repertoire of target antigen-recognition sites (or receptors) on cell surface. Since the primary RCN is pleomorphic, and as the subsequent transplant passages tend to change the histology to monomorphism with reticulum cells

as the dominant feature, it is possible that there are changes in populations of putative suppressor cells or other regulatory cells in the RCN site, which may be reflected to changes in populations of activated NK cells. Further investigation is necessary to elucidate the role of itnerferon and the significance of these observations in terms of immunologic surveillance and evolution of RCN.

d) Studies of immunosuppression by tumor cells and switching on of the host immunosuppressive mechanism by tumor cells.

It has been demonstrated in our previous reports that macrophages can reverse the tumor cell induced suppression in the secondary cell-mediated cytotoxic response against tumor associated antigens, and in the cytotoxic response against alloantigens. Generally it is very difficult to induce cytotoxic response against tumor associated antigen(s) in the syngeneic primary mixed lymphocyte tumor cell cultures. We have found that this is also due to the immunosuppressive effect of the tumor cells which can be overcome by the addition of exogenous, syngeneic peritoneal macrophages. These findings confirm that macrophages play a critical role in the induction of specific tumor immunity.

We have found that tumor cels can trigger the host's own suppressor mechanism via macrophages. In the experiments performed with allogeneic mixed lymphocyte culture reactions or allogeneic mixed tumor cell culture reactions, a nonsuppressive dose of tumor cells can switch on a suppressor mechanism through the collaboration of two populations of macrophage. One is derived from spleen and the other is derived from peritoneal cavity. This observation is further extended to the study of the development of specific tumor immunity in the syngeneic mixed lymphocyte tumor cell culture reactions. These findings suggest that tumor cells may utilize the host's own immune defense mechanism to evade host's immune surveillance network.

e) Molecular cloning of a rat leukemia virus DNA

We have been focusing efforts on the evolution of a transforming DNA sequence. It seemed apparent that DNA recombination between a rat leukemia helper virus (RHHV) DNA and the endogenous Src DNA had resulted in the production of a rat transforming virus KSV (RHHV) isolated in this laboratory. During the past year the DNA sequences specific for (8.4 kbp) RHHV and also for KSV (6.4-7.0 kbp) had each been molecular cloned via the plasmid vector pBR 322 in EK-1 host (E. coli RRI) at the restriction endonuclease site of Bam H1, coincident with the tetracycline resistance locus. Cloning efficiency of 3/5745 and 11/3820, positive in hybridization against virus ^{32}P-cDNa, was observed for RHHV DNA and KSV DNA respectively. The cloned RHHV DNA was analyzed extensively and a restriction endonuclease map of the cloned viral DNA was constructed. Functional organization of cloned DNA was also constructed based on the appearance of S^{35}-methionine labeled cell-free protein products encoded by the mRNA hybrid-selected by the restricted fragments of the cloned RHHV DNA. Ongoing research interest is concentrated on the nucleotide sequencing of the cloned RHHV genomic DNA.

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PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Factors Influencing the Induction, Growth and Repression of Neoplasms and Transfection

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	L.J. Old	Staff Member	Sloan-Kettering Cancer Ctr.
	Nancy Hopkins	Staff Member	MIT Cancer Res. Ctr.
	G. DuBois	Sr. Staff Fellow	LCBGY NCI
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SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: Major emphasis is placed upon the study of tumor antigens of the transplantation rejection type (TSTA), and of tumor antigens (TA) assayed by in vitro techniques and of the immune responses they evoke. As a corollary to this study the biologic properties in vitro and in vivo of histocompatibility (H-2) antigens are under study. Solubilization and methods of purification of both TSTA and H-2 are under investigation with the ultimate purpose of defining these membrane antigens after purification in physico-chemical, biologic and molecular terms. The role of a B-tropic retrovirus as a vector in the transmission of genetic information of a specific tumor antigen (TSTA of Meth A) is under study as well as the use of TSTA markers (Meth A) in the hope of defining more clearly the molecular events in transfection studies using DNA of chemically-induced sarcomas.

I. Tumor Antigens

1. Studies of purification and biological effects of TSTA

A. Attempts to purify to homogeneity the tumor rejection antigen(s) of the Meth A sarcoma. These are collaborative studies (DuBois, Appella and Law, LCB) and DeLeo (Sloan-Kettering). These studies are now made somewhat easier by our recent finding that the majority of TSTA is found in the cytosol fraction of the Meth A cell and one need not resort to solubilization of the cell membranes with detergents. The results of our recent studies with the cytosol-derived antigen(s) are as follows: 1) the majority of the TSTA is in the cytosol (but is found also on the membrane), 2) the biochemical properties, that is, behavior in gel chromatography, lectin affinity chromatography, responses to proteases, etc. and the immunologic properties such as dose-response to antigen, specificity, lack of any evidence of immune deviations in the host are similar in both the membrane-derived and the cytosol derived TSTA of Meth A and also of a recently derived MC-induced sarcoma, CI-4. 3) The active fraction following S-200, lectin and AcA54 chromatography is in the 60-70,000 range and 4) this most enriched cytosol fraction is found also to absorb inhibition of the Meth A specific antiserum that detects TSSA on Meth A cells. Thus TSTA and TSSA appear to be related antigens and these both appear not to be integral membrane proteins but to be peripherally bound to the membrane, and 5) two-dimensional gel electrophoresis of the most active fraction from the final AcA54 chromatography shows that a major component of 68,000 M_r, may represent the TA (TSTA and TSSA).

The most enriched material has been used to develop hybridoma anti-Meth A specific cytotoxic and also precipitating antisera which are being used in further purification.

More recent attempts this year using hydroxyapatite columns and further studies with the main components obtained by 2-dimensional gel electrophoresis now show that the 68,000 MW material is extremely unstable and that a 72,000 MW major band is in all likelihood the active tumor rejection material; curiously the hybridoma antibody against Meth A does not react with the 72,000 MW major band material. One of the stumbling blocks encountered throughout this study is the severe sensitivity of these most purified materials to proteases (see Appella, Annual Report).

B. Another approach to purification of TATA is being used (with Rogers, LCB). Meth A and CI-4, another MCA-induced BALB/c sarcoma of recent origin (also in ascitic form), will both be purified simultaneously from the cytosol using high salt and dithiothreitol to prevent aggregation. Purification has proceeded through extraction and gel filtration and we find that CI-4 behaves like Meth A in that: the soluble (not solubilized) cytosol contains

most of the TSTA activity which is specific for CI-4 and behaves similarly to the membrane-derived TSTA. Small amounts of material (~10µg) give complete protection against CI-4 challenge.

Antisera have been obtained from rabbits. It is the hope that the impurities present at this stage will be common to Meth A and CI-4 and that absorption of the impurities against CI-4 may be used to absorb the impurities against Meth A (and vice versa) leaving TSTA as the unique protein (see Rogers and Law, Int. J. Cancer (in press, July, 1981).

In collaboration with M. Rogers, the immunogenicity and therapeutic efficiency of soluble partially purified CI-4 TATA bound to artificial membranes (egg lecithin liposomes) have been studied. Preliminary results reveal 1) the liposome-bound CI-4 TATA is more effective than CI-4 alone and 2) the CI-4 liposome material is very effective if given as long as 5 days in 5 µg-50 µg amounts prior to challenge with CI-4.

2. Phenotypic studies of cells transfected with high molecular weight DNA from Meth A sarcoma cells

Our cooperative study with the Sloan-Kettering group (Drs. DeLeo and Old) and with Dr. Nancy Hopkins (MIT Cancer Center), concerning the phenotypic expression of Meth A specific tumor antigens (TSTA and TSSA) continues and has yielded interesting results. BALB/c 3T3 (f7) subline, specifically cloned and selected) have been transfected with high molecular weight DNA from Meth A sarcoma and appropriate controls. The following assays done blindly have been accomplished from transformed foci:

1) assay for malignancy

2) presence of TSSA by absorption of the specific cytotoxicity of a Meth A syngeneic antiserum, and

3) tumor rejection and specificity

We have observed a high frequency of co-transfection of transformation and TSTA. All transfected lines (from Meth A DNA) grew progressively in BALB/c mice; 5 of 8 foci selected from one experiment possessed Meth A TSTA. DNA extracted from one of these lines was used in secondary rounds of transfection; 4 of 5 of II transfectants contained Meth A-specific TSTA. These results suggest that in the Meth A sarcoma a transforming gene and a genetic determinant of TSTA are intimately related since one would expect the frequency of a transfer of the 2 phenotypes to be only 0.01 to 0.001 if specified by unlinked genes.

Some of the transfected clones also absorbed cytotoxicity of the Meth A antiserum but this assay appears less sensitive than the in vivo TSTA assay.

One of the transfected lines (BMA-11-7a) has been passaged continuously in vivo and assayed through 15 generations for immunizing capacity against itself and against Meth A. Results show both phenotypes to be stable.

Some of the Meth A DNA cleaved with restriction enzymes retain transforming capacity (Hopkins and Vande Woude); these are now being assayed for the presence of Meth A TSTA. This DNA fragment appears to be of a size (15 Kb) appropriate for molecular cloning. A report of this work has been submitted for publication (Hopkins, Besmer, DeLeo and Law "High frequency co-transfer of the transformed phenotype and a TSTA using DNA from the MC-induced Meth A sarcoma of BALB/c mice").

This investigation is continuing in the following aspects: 1) assays for conversion to malignancy in the transformed foci (transformed in tissue culture) and assays for the TSTA phenotype in the "cut DNA" series, 2) use of another MC-induced sarcoma (CI-4 or CII-10) for transfection studies to determine the generality of these transfections.

A real possibility exists therefore of an assay that will allow the transfecting genes to be cloned with resultant understanding of the chemical carcinogenesis mechanisms.

3. Attempts to isolate and characterize the 11A antigen

We continue our studies of the mechanisms involved in the specific Meth A immunogenicity found in the cells of an M-MSV (MuLV)-induced BALB/c sarcoma (Appella, Law, Chang, Wivel, LCB and DeLeo and Old, Sloan-Kettering). It was found originally that a tissue-culture passaged variant of the neoplasm, 11A, was capable of absorbing cytotoxicity of the specific α-Meth A antiserum, the only one (except Meth A) of more than 100 neoplasms, normal tissues and viruses capable of absorption. The 11A variant was found to immunize BALB/c mice in a specific manner against Meth A only. The 11A variant is a virus producer line but in contrast to the original 11A tumor line (carried in vivo) does not contain rescuable MSV.

An endogenous B-tropic retrovirus has been isolated from the 11A variant line. This uncloned virus (11A-MuLV) when infecting SC-1 cells was found to transmit specific immunity to Meth A. 11A-MuLV was cloned by twice limiting dilutions but upon infectivity of SC-1 cells was negative in specific immunogenicity

assays against Meth A suggesting that 11A-MuLV represents a minority among the 11A-MuLV population of viruses. Then, SC-1 cells were infected with a higher multiplicity of virus (sucrose-banded virus). The infected SC-1 cells were cloned and assayed for XC and RT reactivities and for their ability to absorb cytotoxic α-Meth A antibody. Among the many clones assayed and those showing XC (+) reactivity, about 20% (9 clones) were positive for absorption. These (+) clones assayed for Meth A immunogenicity and only 2 clones (#s 36, 46) were immunogenic against Meth A. Recently however, 2 11A-infected clones (#s 17 and 22) were found to be XC-, RT+ and absorbed Meth A cytotoxicity. These clones produced virus particles and immunized against Meth A - but only in the early passage (not late passage) generations. If virus and TSTA are coordinately expressed, these results suggest that virus replication is inefficient and that successive cell passages reduce the number of progeny virus. These observations are consistent with other data indicating defective virus. Nonetheless early passages of virus in SC-1 cells may provide the source for isolation of the 11A defective virus.

4. Tumor Antigens of the RBL-5 Lymphoma

Efforts during the past year (in collaboration with Dr. O. Alaba) have been concerned with identifying and characterizing those membrane antigens of the RBL-5 cells that are 1) of the tumor rejection type (TSTA), 2) that induce in vitro cytotoxic lymphocytes and 3) that are recognized using a C'-dependent cytotoxicity assay (^{51}Cr) employing a newly developed syngeneic antiserum.

We have still not been able to biochemically isolate and highly purify the RBL-5 TSTA starting with purified plasma membranes (PM). The purification steps included a) gel filtration (AcA34) from DOC-solubilized PM, b) Lens culinaris lentil lectin affinity chromatography with subsequent elution of bound proteins with 3% α-methylmannoside and c) isoelectric focusing. The active TSTA was found between pH 5.5 and 5.8. SDS-PAGE of this active fraction yielded 3 major bands of 80,000, 46,000 and 21,000. In the absence of SDS only one band could be resolved. This 46,000 MW material, sliced from the gels, however, was not specifically immunogenic for RBL-5.

Of interest is the finding that AcA34, fraction 2, containing the bulk of TSTA activity was used to prepare an antiserum by hyperimmunization in syngeneic C57BL/6 mice. This antiserum was found to be specific for RBL-5 but not for any other F, R or M-MuLV induced lymphomas in a ^{51}Cr cytotoxicity assay; absorption assays also proved specificity. Coupling of the gammaglobulins from this antiserum to Sepharose 4B facilitated the isolation of an

antigen that inhibited the C'-dependent lysis of RBL-5 cells and immunized specifically in vivo against RBL-5 challenge.

Studies were completed defining the parameters of secondary in vitro generation of syngeneic CTL by solubilized tumor antigens from RBL-5. Use of solubilized antigen run through the affinity chromatograph-Sepharose 4B column was shown to be enriched for specific CTL activity; the specificity of the induction and effector phases was assessed and data clearly indicated that the reactions were specific. It remains to be studied whether the CTL-induction antigen is identical with TSTA (unfortunately Dr. Alaba has accepted a position in Nigeria and the bulk of this work is now held in abeyance).

Significance for Cancer Research

Characterization of tumor antigens of the rejection type is a necessary prerequisite for understanding the mechanisms of immune surveillance, tumor inhibition and facilitation. In addition the role of these membrane bound components in the mechanisms of initiation and maintenance of malignancy will be studied.

As a basis for any study of membrane bound antigens it is necessary to study the nature of histocompatibility antigens and their relationship to tumor antigens. Thus our emphasis is on parallel studies of H-2 antigens in order to provide a basis for understanding tumor antigens.

Objective 3; Approaches 4,5.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03229-12 LCBGY

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure and Cloning of B_2 Microglobulin and Mouse Histocompatibility Antigens.

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SUMMARY OF WORK (200 words or less - underline keywords)

Biochemical and structural studies of histocompatibility antigens, tumor antigens, transformation related antigen (p53) and epoxide hydrase. It is the purpose of this project to study the chemistry and structure of different molecules. Biochemical isolation of histocompatibility antigens, transformation related protein (p53), tumor antigens and epoxide hydrase is carried out. The chemical structure of these proteins is being approached by micro methods and the relationship of the amino acid sequence to other known structures is being analyzed. Cloning of histocompatibility antigens, β_2 microglobulin and the p53 protein is being approached in order to study the molecular organization of these systems and permit a better understanding of the nature of the extensive polymorphism at the H-2 loci and the mechanism involved in the regulation and expression of different genes.

HISTOCOMPATIBILITY ANTIGENS

1. cDNA clones encoding mouse histocompatibility antigens

We have obtained cDNA libraries from poly(A)$^+$RNA isolated from ascites cells of one inbred strain of mice (LSTRA) and screened the libraries with a cDNA clone encoding a human transplantation antigen. Three cDNA clones were identified. One clone was sequenced and found to encode amino acids 50-287 of an H-2 like antigen with 90% homology to the published protein sequence of H-2K^b. Comparison of this cDNA sequence with both β_2-microglobulin (β_2M) and the constant region domains of mouse μ immunoglobulin gene revealed a striking homology which suggested that the three genes shared a common ancestor. Restricted enzyme digests of liver DNAs from different inbred strains of mice were hybridized to such probes and 10-15 bands were found for each strain; these results indicate that the histocompatibility antigens appear to constitute a multigene family.

2. Control of the expression of mouse histocompatibility antigens and β_2M in F9 teratocarcinoma stem cells

F9 teratocarcinoma stem cells differ from most other cell types; these cells do not express any major histocompatibility antigens. Evidence has been obtained that this regulation of major histocompatibility antigen expression is due to transcriptional control of the major histocompatibility antigen genes (H-2 and β_2M). The steady state levels of β_2M and H-2 mRNA from F9 teratocarcinoma stem and differentiated cells were examined by "Northern Blot" hybridization using cloned DNA probes specific for these mRNAs. F9 teratocarcinoma stem cells (clone 12-1) contain at least ten fold less H-2 and β_2M mRNA than the differentiated daughter cells (clone 12-1a).

The transcriptional regulation of these genes is accompanied by a change in their DNase I sensitivity. Normally transcriptionally inactive genes are DNase I resistant, while active genes are DNase I sensitive. In contrast, the silent major histocompatibility antigens of teratocarcinoma stem cells are more DNase I sensitive than the active genes of the differentiated cells. Commitment to H-2 or β_2M expression apparently is not correlated with a chromatin conformation which confers increased sensitivity to DNase I digestion as has been observed in other systems such as globin, adenovirus and ovalbumin genes.

3. Control of expression of H-2 antigens on mouse placenta cells

Because of the placenta's capacity for rapid cell replication, this organ is an excellent model for the study of normal and abnormal

cell replication and for investigating the regulation of gene expression. In our first attempt to use such a system, we have utilized two established cell lines of BALB/c and C57BL/6 origin which appear to be trophoblasts as judged by their production of gonadotropin-like substance and steroid hormones. These cell lines do not express H-2 and β_2M on the membrane. They synthesize only very low amounts of the two proteins. Analysis of the mRNAs present by hybridization to specific probes revealed that substantial amounts of the RNAs are present. The defect in expressing H-2 and β_2M is therefore at the translational level. Further work is being carried out to assess the molecular events which are involved in the regulation of the expression of these genes.

4. Studies on a murine ovarian teratocarcinoma that lacks histocompatibility antigens

A murine ovarian tumor in C3HeB/FeJ mice provided an excellent model for studying the biologic behavior of human ovarian cancer. We were unable to generate either allogeneic or syngeneic cytotoxic cells capable of lysing the tumor by in vitro immunization. The absence of biologically significant levels of histocompatiobility antigens was noted in both antibody and cell mediated cytotoxicity assays. Immunochemical analysis of radiolabeled tumor extracts failed to demonstrate a H-2 heavy chain molecule, despite the presence of a normal amount of β_2 microglobulin in the same extracts. Thus this murine ovarian tumor which has a serologically detectable tumor-associated antigen (Order et al., Cancer (Phila.) 34, 175-183, 1974) and can be cured by non-specific immunotherapy, may provide an excellent model for the study of successful immunotherapy in the absence of histocompatibility antigens and associated cell mediated reactions.

5. Purification and characterization of mouse β_2 microglobulin variants from two different strains

Variants of β_2M (β_2m^a (BALB/c strain) and β_2m^b (C57BL/6 strain) first detected by Michaelson et al. (Immunogenetics 11: 93-95, 1980) have been purified to homogeneity in reproducible yields (10-12%) using an improved method. The purifications were accomplished by a 3M sodium thiocyanate extraction of a crude membrane fraction, an acid precipitation step, gel filtration on Sephadex G-75 and ion exchange chromatographies on DEAE-cellulose and CM-cellulose respectively. Charge differences observed between the two variants on ion-exchange columns were consistent with differences in pI values and in amino acid composition. β_2m^a was found to more acidic (pI = 7.35) than β_2m^b (pI=7.65). β_2m^a contained 10 aspartic acids and 3 alanine residues while β_2m^b contained 9 aspartic acid and 4 alanine residues; no other differences in the amino acid composition were observed. Structural evidence for the strain related polymorphism of β_2M was obtained by a) determination of the primary structure of β_2M and b) partial sequence analysis of β_2m^b. Complete sequence analysis of β_2m^a was performed by an

automated Edman degradation of the intact chain and of the overlapping peptides obtained by (a) tryptic cleavage at arginines after acetimidation of lysines; b) BNPS-skatole cleavage at tryptophan and c) hydroxylamine cleavage at asparagine-glycine linkages. A comparison of the primary structure of β_2m^a to the partial sequence obtained for β_2m^b revealed that a single amino acid substitution (aspartic acid to alanine at position 85) accounts for the biochemical differences observed.

6. Mapping the gene for β_2M in the mouse using somatic cell hybrids

The segregation of mouse chromosomes in mouse-hamster somatic cell hybrids has been demonstrated by assaying for enzymatic markers for the different mouse chromosomes. The ability of radiolabeled clones to synthesize mouse β_2M has been tested by immunoprecipitation and two-dimensional gel electrophoresis. Approximately 40 clones have been examined. Near perfect concordance of one of the enzyme markers with the β_2M function has made it possible to assign the β_2M gene to mouse chromosome 2. Enzyme segregation analysis excluded all chromosomes for β_2M assignment except chromosome 2. Karyotype analysis of 8 informative hybrid clones confirmed the assignment of β_2M to mouse chromosome 2.

7. Mouse β_2M cDNA clones

We have isolated three cDNA clones for β_2 microglobulin. β_2 microglobulin comprises less than 0.1% of mouse liver protein, and its mRNA is approximately 0.03% of liver poly(A)$^+$ mRNA. The cDNA clones were identified by screening 1400 cDNA clones made from 9-10S mouse liver poly(A)$^+$ mRNA. The procedure for screening the cDNA clones involved binding pooled plasmid DNA to nitrocellulose filters and testing the ability of each filter to select β_2M mRNA. The filter selected mRNAs were assayed for their ability to direct the synthesis of β_2M in *in vitro* translation reactions. The isolated clones were shown by nucleotide sequence analysis to encode β_2M. The positive-selection hybridization assay has been modified to facilitate the screening of large numbers of cDNa clones, and should allow the isolation of cDNAs corresponding to any mRNA whose *in vitro* translation products can be immunoprecipitated. The cDNA clones for β_2M contain sequences corresponding to the entire β_2M mRNA with the exception of the 5' and part of the 3' untranslated regions and the regions encoding the signal peptide amino acids 1-10 and residues 42-49. These clones provide nucleic acid probes to examine the structure of the β_2M gene(s). Preliminary characterization of mouse β_2M gene sequences by Southern blot analysis suggests that there is only a single complete β_2M gene. This gene has two intervening sequences located near the ends of the coding sequence.

8. Purification and biochemical characteristics of a β_2 microglobulin associated protein from mouse serum

Normal mouse serum has been found to contain a high molecular weight fraction containing β_2M and lacking H-2 alloantigenic activity (Kirst and Peterson, Biochemistry 17, 4794 (1980); Natori et al. J. Immunogenet. 3, 123 (1976). Partial characterization of this protein on a rabbit anti-H-2 immunoabsorbent column (Kirst and Peterson, Biochemistry 17, 4794 (1980) revealed the presence of a 42,000 MW component tightly associated with β_2M. Comparison of peptide maps between purified H-2 and this serum protein indicate a very low degree of homology (Robinson, E., Tanigaki, T., Appella, E., unpublished results). We have now undertaken to purify the intact β_2M associated protein from mouse serum in order to obtain information abut its biochemistry and functions. First, sequence analysis of the first 20 residues will indicate whether it is structurally related to H-2 or not. Second, information about its synthesis and regulation would lead to genetic mapping. The data obtained from such experiments would give information about a probable biological role for β_2M and the structural features needed for non-covalent interaction with disparate structures.

9. T-cell activation: evidence for a dual receptor model

The immune system is a complex collection of cells capable of responding to virually any foreign molecular configuration. Among these cells, the T-cell plays an important role. We have been interested in defining the T-cell receptor and used the cytochrome system. In this system the C-terminal cyanogen bromide cleavage fragments of cytochrome c ('C') and their acetimidyl derivatives (Am-'C') form two families of antigens. The native and derivatized fragments are antigenically distinguished in both B10.A and B10.A(5R) mice. Genetic studies indicate that the T-cell proliferative responses to both antigenic families utilize only the E:A restriction element. Nonetheless, the pattern of cross-reactivity within each family is unaffected by the derivatization. Thus, B10.A nylon wool passed lymph node cells (NWPLN) primed in vivo with pigeon 'C' or pigeon Am-'C' show heteroclitic stimulation in vitro with tobacco horn worm moth (THWM) 'C' or THWM-Am-'C' respectively, and both show weak or absent stimulation with duck 'C' or duch Am-'C'. However, B10.A(5R) NWPLN cells primed to pigeon Am-'C' show a pattern of strong stimulation with duck Am-C and are not heteroclitically stimulated by THWM-Am-'C'. These patterns are evidently controlled by genes in the major histocompatibility complex (MHC) since B10.A and B10.A(5R) differ. These data are most readily explained by hypothesizing two antigen "receptors": one common to both families to account for their common patterns of cross-reactivity; a second which differs between the two families to account for their antigenic distinctness. Experiments with synthetic peptides are under way to

determine which residues effect the distinction between native and derivatized peptides and which effect the cross-reactivity patterns. Experiments with somatic fusions between BW5147 and antigen-stimulated NWPLN cells are underway to determine 1) the cellular location of each receptor and 2) whether complementation between independently primed receptors in the secondary response is possible. These experiments potentially can distinguish between dual receptor and single receptor models of MHC-restricted T cell antigen recognition.

TUMOR ANTIGENS

1. The soluble tumor antigens of BALB/c sarcoma Meth A

The cytosol of the BALB/c sarcoma Meth A contains both a serologically defined tumor specific surface antigen (TSSA) and a tumor associated transplantation antigen (TATA). The relationship between these two antigens has been investigated. Chromatographic fractions derived from the cytosol of Meth A cells which possess a soluble TATA were assayed for the serologically defined TSSA by absorption analysis. Meth A cells were lysed and a high speed supernatant (HSS) prepared. This HSS was treated with 1.25% streptomycin sulfate and nucleic acids were removed by centrifugation. The supernatant was brought to 55% $(NH_4)_2SO_4$ saturation and the protein pellet dialyzed overnight. The dialysate was then applied to a hexylamine-agarose column and eluted with a gradient of 0-1.0 M NaCl. Four main fractions were eluted from the column. The majority of the TATA activity (80%) was recovered in the unabsorbed fraction (MHo). The MHo fraction was found to be specific in the in vivo tumor rejection assay. The MHo fraction was further chromatographed on an Ultrogel AcA44 column. Five fractions (F_I-F_V) were recovered which covered a range of molecular weights between 170K and 10K. The high molecular weight fraction, F_I (~170K) contained 80% of the original activity while F_{II} (~67K) contained about 10% of the activity. The first four fractions (F_I-F_{IV}) were assayed for the serologically defined TSSA by absorption analyses; only F_1 which was enriched 25 fold in TATA showed complete absorption of cytotoxicity. Furthermore the absorption was specific only for the Meth A antiserum and not for a similarly prepared antiserum defining a TSSA on the CMS4 tumor. The AcA44-F_1 fraction was chromatographed on hydroxylapatite to give five fractions HA-1A, 1B, 2,3 and 4. All HA fractions were tested in the in vivo tumor rejection assay. Only HA-2 (major component 65K, pI 5.8) and HA-4 (major components 72K and 76K) gave substantial inhibition of tumor growth. When these same fractions were assayed by absorption analysis, only HA-2 showed partial absorption of cytotoxicity. The AcA44-F_I fractions was also subjected to preparative SDS polyacrylamide gel electrophoresis. This technique

yielded highly purified fractions of the 65K, pI 5.8 component and the 76K pI 6.3 component. At doses of 10 µg in carrier BALB/c serum, the 65K and 76K protens both provided complete protection against Meth A tumor growth. Thus our studies to date indicate that there are two proteins possessing TATA activity. The 65K component is extremely sensitive to proteolysis and this fact suggests that it may be a lower molecular weight form of the 76K protein. The exact relationshp between these two proteins is currently under study. The purification and characterization of a discrete TATA now allows the exciting exploration of the relationship between the genetic determinants of transformation and a unique TATA of Meth A sarcoma.

2. p53, a transformation-related protein

Malignant cells of the mouse or other species, whether transformed by viruses, chemicals or x-ray, express a cellular protein with a subunit molecular weight of 53,000. Detectable levels of p53 were not found in a broad range of normal cell types. A 53,000 molecular weight protein has been detected in transformed B cells of human origin (Luka, Jornvall and Klein, J. Virol. 35, 582, 1980) but not in non transformed B cells. In cell lines carrying the Epstein-Barr virus-directed EBNA antigen, the p53 component forms a complex with EBNA. The nature of the regulatory defect that leads to persistent high levels of p53 in transformed cells is an important question. In order to answer this question we have attempted purification of the protein in order to characterize its structure and construct nucleic acid probes that detect the p53 gene. Our preliminary results (Jornvall, Luka, Klein and Appella, unpublished results) indicate that purified p53 obtained from mouse or human transformed cell lines have very close amino acid compositions and sequences of the first 20 residues. Further work now in progress will be directed toward the elucidation of the complete amino acid sequences in order to understand the function of this protein and its role in the regulation of cell division.

PROTEIN CHEMISTRY

1. The amino acid sequence of rat liver epoxide hydrase

Previous attempts at chemical cleavage of purified rat liver epoxide hydrase by reagents specific for methionine or typtophan have failed to produce peptides suitable for subsequent sequence studies. However, epoxide hydrase has now been cleaved specifically by means of an acid sensitive, aspartyl-proline linkage to give excellent yields of two peptides, one a 33K peptide and the other a 17K peptide. The reagent employed was 70% formic acid in 7M guanidine hydrochloride and cleavage was complete after 72 hours. The improved cleavage of the main polypeptide chain of epoxide hydrase will greatly facilitate subsequent protein sequence studies.

2. Structure of the adenovirus hexon protein

The hexon protein is the main capsid protein of adenovirus. Studies on its primary structure have been completed. It consists of 966 amino acids and the primary structure was obtained by a combination of both protein and DNA sequence analysis. These data are important since crystallographic analyses are now in progress and have provided an understanding of the position of critical amino acid residues in relation to conformation and antigenic site structure.

Publications:

Parnes, J.R., Velan, B., Felsenfeld, A., Ramanathan, L., Ferrini, U., Appella, E. and Seidman, J.G.: Mouse β_2-microglobulin cDNA clones: A screening procedure for cDNA clones corresponding to rare mRNAs. Proc. Natl. Acad. Sci. USA 78: 2253-2257, 1981.

Croce, C.M., Linnenbach, A., Huebner, K., Parnes, J.R., Margolies, D., Appella, E., and Seidman, J.: Control of the expression of histocompatibility antigens (H-2) and beta-2 microglobulin in F9 teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA, in press, 1981.

Cox, D.R., Sawicki, J.A., Goldblatt, D., Appella, E., and Epstein, C.J.: Assignment of the gene for β 2-microglobulin to mouse chromosome 2. J. Exp. Med., in press, 1981.

Vanhaelen, C.P.J., Fisher, R.I., Appella, E., and Ramanathan, L.: Lack of histocompatibility antigens on a murine ovarian teratocarcinoma. Cancer Res., in press, 1981.

Hansburg, D., Hannum, C., Inman, J.K., Appella, E., Margoliash, E., and Schwarz, R.H.: Parallel cross-reactivity patterns of two sets of antigenically distinct cytochrome C peptides: possible evidence for a presentational model of IR gene function. J. Exp. Med. in press, 1981.

DuBois, G.C., Appella, E., Law, L.W., DeLeo, A.B., and Old, L.J.: Immunogenic properties of soluble cytosol fractions of Meth A sarcoma cells. Cancer Res. 40: 4204-4208, 1980.

DuBois, G.C., Appella, E., Law, L.W., DeLeo, A.B., and Old, L.J.: The soluble antigens of BALB/c sarcoma Meth A: A relationship between a serologically defined tumor specific surface antigen (TSSA) and the tumor associated transplantation antigen (TATA). Transplantation Proc., in press, 1981.

Jay, G., DeLeo, A.B., Appella, E., DuBois, G.C., Law, L.W., Khoury, G., and Old, L.J.: A common transformation-related protein in murine sarcomas and leukemias. Cold Spring Harbor Symp. Quant. Biol. XLIV: 659-664, 1980.

Inman, J.K., DuBois, G.C., Appella, E., and Perham, R.N.: Use of aqueous methylamine for rapid cleavage of amidine linkages to proteins and peptides treated with imidoesters. In Liu, D. and Schechter, A. (Eds.), The Synthesis and Sequencing of Peptides and Proteins, New York, Elsevier/North Holland, Inc., May, 1981.

Jornvall, H., Appella, E., Fowler, A., and Von Bahr-Lindstrom, H.: Structural analysis of four large CNBr fragments from ^{14}C-carboxymethylated adenovirus hexon protein. J. Biol. Chem., in press, 1981.

Jornvall, H., Akusjarvi, G., Alestrom, P., Von Bahr-Lindstrom, H., Pettersson, U., Appella, E., Fowler, A.V., Philipson, L.: The adenovirus hexon protein: the primary structure of the polypeptide and its correlation with the hexon gene. J. Biol. Chem., In press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 32555-06 LCBGY

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immune Response to Tumor Associated Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.C. Ting	Medical Officer	LCBGY NCI
OTHER:	J.X. Hong	Guest Worker	LCBGY NCI

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LAB/BRANCH
Laboratory of Cell Biology

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Office of the Chief

INSTITUTE AND LOCATION
NCI,NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	2.50	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Switching on by tumor cells of the macrophage-mediated suppressor mechanism to evade host immune surveillance: Tumor cells can trigger the host's own suppressor mechanism via macrophages to suppress the T cell-mediated cytotoxic response. This is demonstrated in the allogeneic mixed lymphocyte culture reactions and allogeneic mixed lymphocyte tumor cell culture reactions in studying the immune response against alloantigens, and in the syngeneic mixed lymphocyte tumor cell culture reactions in studying the immune response against tumor associated antigens. It appears that tumor cells can utilize the host's own immune system to evade the host's immune surveillance.

2. T and B cell collaboration in lymphoproliferative response to tumor cells: Proliferative response to a methylcholanthrene tumor Meth A in syngeneic BALB/c mice is primarily mediated by B cells. However, in order to obtain an efficient response, helper T cells are required, as shown by a 2-5 fold of augmentation of this B cell response in the presence of 10-50% T cells.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives. 1) Immunosuppression by tumor cells and reversal of immunosuppression; 2) switching on the host immunosuppressive mechanism by tumor cells; 3) immune response to tumor associated antigens; 4) T and B cell collaboration in immune response to tumor associated antigens.

Methods employed. Tumor cells transformed by oncogenic viruses, chemicals or unknown agents are kept in tissue culture or as transplanted tumors in mice. The ^{125}IUdR release assay was used to measure the cell-mediated cytotoxic response in vitro, and the adoptive transfer experiment was used to measure the cell-mediated immune response in vivo. Lymphoproliferative response was determined by ^{125}IUdR incorporation. Humoral antibody response was determined by complement dependent cytotoxicity test and isotopic antiglobulin assay.

Major Findings:

I. Immunosuppression by tumor cells and reversal of immunosuppression by macrophages: It has been demonstrated in our previous reports that macrophages can reverse the tumor cell induced suppression in the secondary cell-mediated cytotoxic response against tumor associated antigens, and in the cytotoxic response against alloantigens. Generally it is very difficult to induce cytotoxic response against tumor associated antigen(s) in the syngeneic primary mixed lymphocyte tumor cell cultures. We have found that this is also due to the immunosuppressive effect of the tumor cells which can be overcome by the addition of exogenous, syngeneic peritoneal macrophages. These findings confirm that macrophages play a critical role in the induction of specific tumor immunity.

II. Switching on by tumor cells of the macrophage-mediated suppressor mechanism: We have found that tumor cells can trigger the host's own suppressor mechanism via macrophages. In the experiments performed with allogeneic mixed lymphocyte culture reactions or allogeneic mixed lymphocyte tumor cell culture reactions, a nonsuppressive dose of tumor cells can switch on a suppressor mechanism through the collaboration of two populations of macrophage. One is derived from spleen and the other is derived from peritoneal cavity. This observation is further extended to the study of the development of specific tumor immunity in the syngeneic mixed lymphocyte tumor cell culture reactions. These findings suggest that tumor cells may utilize the host's own immune defense mechanism to evade host's immune surveillance network.

III. Collaboration of T and B cells in the lymphoproliferative response to tumor cells: High levels of proliferative response to Meth A tumor cells (a methylcholanthrene induced sarcoma) can be induced in syngeneic BALB/c mice. This response is primarily induced by B cells. The responders are found in the enriched B cell preparation but not in the T cell preparation. Nude mice are also able to mount a significant proliferative response. However,

presence of 10-50% of T cells can greatly augment this B cell-mediated proliferative response, indicating that collaboration of T cells are required for the induction of an efficient response.

IV. Development of tumor immunity in tumor bearing hosts: Specific and nonspecific cell-mediated cytotoxic response can be demonstrated at the tumor site in mice with progressively growing tumors and in mice with regressor tumors. Specific cytotoxicity is mediated by T cells, and nonspecific cytotoxicity is mediated by non T cells. It is found that only T cells appear to be able to confer in vivo tumor immunity.

Significance for biomedical research and the program for the National Cancer Institute: Development of an efficient immune response against tumor cells involves a complex series of interactions between different populations of lymphocytes, macrophages and humoral mediators. Therefore, we need to understand how do tumor cells suppress the host immune system and how does the host counteract with tumor cells with its various immune networks. It is only through the combined efforts to study the various aspects of in vitro immune response and their in vivo counterparts, that we shall understand how can host develop an efficient immune response to eliminate the tumor cells.

Proposed Course:

I. To further study the tumor cell triggered macrophage-mediated suppression, both in murine models and in human system.

II. To study the development of cell-mediated cytotoxicity at the tumor sites, and its correlation to the development of in vivo tumor immunity concerning progression and regression of tumor growth.

III. Collaborative study on tumor immunology and cancer epidemiology with scientists in the People's Republic of China.

Publications:

Ting, C.C., and Rodrigues, D.: Switching on by tumor cells of the macrophage-mediated suppressor mechanism to evade host immune surveillance. Proc. Natl. Acad. Sci. USA. 77: 4265-4269, 1980.

Ting, C.C., Rodrigues, D., and Hong, J-X. Characterization of the responding populations for the generation of proliferative response to syngeneic Meth A tumor in BALB/c mice: Requirement of T and B cell collaboration. J. Immunol. 125: 2742-2748, 1980.

Rodrigues, D., and Ting, C.C.: Studies of the mechanisms for the induction of in vivo tumor immunity. V. A comparison of the generation of the primary cell-mediated cytotoxic response using in vitro mixed lymphocyte tumor cell culture and an in vivo technique. Cellular Immunol., in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 04833-12 LCBGY

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Biological Studies of Various Normal, Virus-Infected, and Malignant Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	S.S. Yang	Chemist	LCBGY	NCI

COOPERATING UNITS (if any)
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E. Appella, LCBGY, NCI

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the primary purpose of this project to study some of the pertinent factors which influence cell differentiation and malignant transformation, using techniques and approaches which range from the microscopic to the molecular level. Particular emphasis is given to those systems in which murine RNA tumor viruses or their precursors or chemical carcinogens may be the transforming agent. A variety of mouse model systems are used, including plasma cell tumors, mammary tumors, neuroblastomas, and methylcholanthrene-induced sarcomas. Current projects include: 1) effects of interferon on the assembly and maturation of murine retroviruses with special emphasis on the study of mechanisms whereby whole virions are rendered noninfectious; 2) effects of interferon on methylcholanthrene-induced sarcomas of the BALB/c mouse with the aim of defining anticellular and/or immunological activity; 3) relationship of retroviruses to the expression of certain phenotypic changes in chemical carcinogen-induced murine sarcomas.

PHS-6040
(Rev. 2-81)

Major Findings:

1. Effects of interferon on murine retroviruses. Most of the studies in this system are being done with Dr.Paula Pitha and reflect a continuing interest in the molecular mechanisms which account for the effect of interferon on retroviruses. In the case of most viruses, interferon causes an impaired mRNA translation with resulting defects in viral structural proteins. However, the effects of interferon on retroviruses occur after the synthesis of viral RNA and most of the structural proteins. It is now well established that the predominant mode of action concerns the late stages of virus assembly and maturation. Further, the precise type of change observed seems to vary among the different classes of retroviruses. Yet the end result is essentially the same, the production of particles which are noninfectious. It must be emphasized that this interference with assembly is dependent on the continuous presence of interferon in the in vitro system; following withdrawal of interferon there is an almost complete recovery of virus titer within 18-24 hours. In the case of ecotropic viruses such as Gross and Rauscher murine leukemia virus, there is maturational arrest at either the budding or release stage depending on the type of host cell used for propagation. These ultrastructural observations correlate with a decreased rate of proteolytic cleavage involving the high molecular weight polyprotein precursors of the env and gag gene products. As a result the noninfectious particles contain env pr85 and gag Pr65 as well as gp71 and p30.

Our most recent experiments have been done with a new class of murine retrovirus, MCF, which is apparently an env gene recombinant of ecotropic and xenotropic viruses endogenous to the AKR mouse, and which seems to accelerate the development of leukemia. One of the characteristics of MCF virus is a slow rate of processing of the pr76 env precursor to gp71, as compared to the ecotropic viruses. Since previous studies with ecotropic viruses indicated altered rates of proteolytic cleavage of the env gene products, the strategy in using MCF was to exploit an assembly stage in which the kinetics of cleavage are already slower than average. Notwithstanding this initial thesis, the results reflect a more complex situation than originally anticipated.

Chronically infected SC-1 cells were treated with 150 to 600 units/ml of highly purified interferon for 24 to 48 hours, and then the virus was titered by focus assay on mink cells. There was approximately a 100-fold reduction of infectious virus post interferon treatment, but the reverse transcriptase assay indicated only a ten-fold reduction in the number of particles, indicating a significant production of noninfectious virus. Furthermore, there was a 2.5 fold increase in the number of virions accumulating along the cell surface following interferon treatment, suggesting that release is slowed. There were

no differences in the synthesis and processing of viral proteins in the cells in the presence or absence of interferon. The amount of specifically labeled cell surface viral proteins detected in virions produced in interferon-treated cells was dependent on the length of exposure. Virions produced in cells treated with interferon for 24 hours contained less gp71 than the controls and this difference was further accentuated after 48 hours. No decrease in p30 was apparent after 24 hours of treatment, but it was obvious at 48 hours. Virions produced in the presence of interferon for 48 hours were lacking both p15E and p12E, while the control MCF virions contained, preferentially, p12E. Interferon-treated virions contained an additional glycoprotein, gp85, but it could not be immunoprecipitated by viral specific reagents. These results suggest that the synthesis and processing of viral proteins in interferon-treated cells is apparently normal, but the assembly of these proteins into mature virions is significantly altered, resulting in slowed virus release and a marked decrease in infectivity.

2. Effects of interferon on methylcholanthrene-induced sarcomas of BALB/c mice. The rationale for pursuing these studies was predicted on a number of distinct but related factors. First, the clinical literature regarding the interferon treatment of various human sarcomas is less than definitive. Thus one could expect to derive a more interpretable set of data regarding properties of interferon in a controlled animal model system maintained in syngeneic mice. The methylcholanthrene-induced sarcoma, Meth A, meets the aforementioned requirements; additionally it can be serially passed both in vivo and in vitro. There are no known murine retroviruses or other viral agents associated with this tumor and thus one could easily dissect the anticellular effects of interferon from the antiviral ones. It is also of importance that there are a number of stable membrane markers such as the tumor associated transplantation antigen (TATA) and the tumor specific surface antigen (TSSA) which are potentially susceptible to the action of interferon.

The ascites form of Meth A was used to establish a continuous tissue culture line yielding a tumor dose 50 between 10^3 and 10^4 cells. For the challenge experiments, tumor cells were treated in vitro with interferon (1000 units/ml) for 72 hours; this dosage level easily exceeded what could be achieved in the intact animal. At the end of the treatment period, the cells showed essentially 100% exclusion of trypan blue and the cell counts were 90-95% of that seen in the controls. Challenge doses ranged from 3.75×10^3 to 1×10^6 cells and maximal efects were seen at a level of 5×10^4 cells; 81% of control mice developed tumors whereas 19% of mice receiving interferon-treated cells developed tumors. Since interferon treatment showed little inhibition of cell division or DNA synthesis, othe parameters of cell function were evaluated. TATA expression was studied by tumor rejection assay. Immunization was done using two inoculations of 2×10^6 Meth A cells (irradiated with 10,000 R) 10 days apart

and challenge was done with 5 x 10^4 tumor cells. There was 100% tumor rejection after immunization with either control or interferon-treated irradiated cells. In a related experiment, mice which did not develop tumors post-inoculation with interferon-treated cells were rechallenged with control Meth A cells, and there was 100% tumor rejection, indicating that TATA immunization occurred although tumors did not develop. When these same mice were later challenged with MKSA cells, which have a TATA of different specificity, 100% of the mice developed tumors. In sum these data suggest that the major effects of interferon on Meth A are not mediated through anticellular activity. Since there is some evidence indicating that administration of interferon can act as a stimulus or signal for activation of immune cells, current efforts are directed toward the study of interferon-treated Meth A cells in immunosuppressed mice.

3. Relationship of retroviruses to expression of certain phenotypic changes in methylcholanthrene-induced sarcomas. As a class of viruses, the murine retroviruses derive certain properties from the fact that their genes are endogenous to the host. Expression of these genes is most probably related to the alteration of host control mechanisms. Depending on the integration site of these virogenes and depending on what cell sequences are adjacent, it is conceivable that non-viral sequences could be linked to the viral genome, resulting in the co-expression of viral particles and non-viral gene products. Such a hypothesis is being tested experimentally and the preliminary results are of some interest.

After alternate in vivo and in vitro passages, a line of NIH-3T3 cells was observed to have the capacity to absorb the TSTA activity associated with a methylcholanthrene-induced sarcoma. This particular cell line was found to be producing a retrovirus which apparently lacks the capacity to replicate in X-C cells or to transform cells. However, C-type particles are produced and attempts to pass this agent to susceptible cells (SC-1) have been successful. If TSTA and the virus are co-expressed, then injection of non-sarcoma cells could provide a vehicle for eliciting tumor rejection in BALB/c mice. Several clones of infected SC-1 cells have been used in just such a manner with mixed results. Protection against challenge with sarcoma cells is more effective when SC-1 cells are used during the first few passages after infection. If the virus and TSTA are coordinately expressed, these results suggest that virus replication is inefficient and that successive cell passages progressively reduce the number of progeny particles. These observations are consistent with the assumption that the virus is defective, at least in its capacity for efficient horizontal transfer. Further studies characterizing the virus are being actively pursued.

Publications:

Pitha, P.M., Fernie, B., Maldarelli, F., and Wivel, N.A.: Effect of interferon on assembly of MuLV, In D.L. Eisenberg, I.A. Luke and C. Fred Fox (eds.), Biological Recognition and Assembly, New York, Alan R. Liss, Inc., 1980, pp. 101-109.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 04834-05 LCBGY

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Cloning, Organization and Hybrid-select Expression of Rat leukemia virus DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S.S. Yang Chemist LCBGY NCI

COOPERATING UNITS (if any) J. Costa, Head, Surgical Pathol. and Postmorten Section, Lab. of Pathology, NCI, N.A. Wivel, Head, Ultrastructural Biol. Section, LCBGY, NCI, M. Gardner, Dept. of Pathology, School of Medicine, USC, California, Rama Modali, Grad. Student, Howard Univ., Dept. Biochem., Washington, DC

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Ultrastructural Biology Section

INSTITUTE AND LOCATION
NCI,NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major thrust of this study is to elucidate the molecular genetics of neoplastic transformation of normal tissues. Two experimental systems were used: 1) Two rat leukemia helper viruses - a) KSV(RHHV) originally isolated in this laboratory and b) WR-RaLV, a wild rat tumor virus. 2) Intracisternal A particle from BALB/c mammary tumor cell line and plasma cell tumor, MOPC-104E. Multidisciplinary approaches involving nucleic acid and protein chemistry and tissue culture were employed in this investigation. We have earlier completed extensive characterizations morphologically, biologically, biochemically and immunologically of these retraviruses. Our current interests focus on the molecular mechanisms involved in the evolution of a transforming DNA sequence through the recombination between a rat leukemia helper virus DNA sequence and rat endogenous DNA sequence. Our total research efforts are concentrated on: 1) Molecular cloning and recombinant DNA analysis of RaLV and KSV DNA sequences. 2) Restriction endonuclease mapping of the cloned viral DNA sequences, 3) Functional organization of the viral genome, 4) IAP specific DNA sequence in DNA of human chondrosarcoma carried in nude mouse.

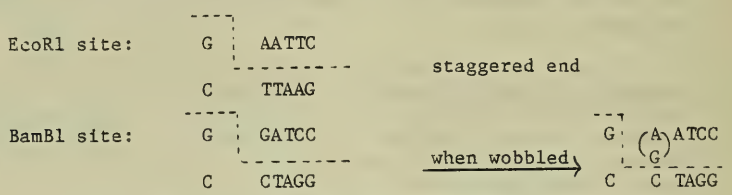
Major Findings:

1. Molecular cloning and recombinant DNA analysis of RaLV and KSV DNA sequences via a plasmid vector pBR 322 in E. coli RRI host

It has been proposed that murine type-C leukemia virus (MuLV) generally of low pathogenicity, when recombined with a subset of host cellular nucleotide sequences (Src genes), give rise to competent oncogenic viruses. The laboratory isolates of rat RNA tumor viruses with transforming activity such as Harvey sarcoma virus (HaMuSV) and Kirsten sarcoma virus (K-MuSV) have been considered such recombinants based on homologous oligonucleotide distribution patterns to rat cellular 30S RNA and MuLV genomic RNA. Recently three strictly rat-tropic RNA tumor viruses have been isolated, namely hepatoma rat leukemia helper virus (RHHV) isolated in our laboratory, wild rat leukemia virus (WR-RaLV), and Sprague-Dawley rat leukemia virus (SD-RaLV). These were considered as endogenous rat type-C viruses since they were released from chemically induced tumors. Upon recombination with the Src sequences some of these have demonstrated cell transformation capacity. The analyses of the genomic complexity of these endogenous rat leukemia viruses would no doubt provide further insights on the "helper" sequence and its functional organization with respect to the Src sequences in the genome of a transforming rat virus.

Relatively little is known of the genomic organization of the endogenous rat leukemia helper viruses although extensive and comprehensive analyses of the structural and functional organizations of both the Ha-MuSV and K-MuSV have been documented. Recently we have identified the DNA sequences specific for both the endogenous RHHV and WR-RaLV as 8.0-8.8 kilobases in length. Among the endogenous ecotropic MuLV of the AKR type and the Mo-MuLV, genomic DNA also shows a larger size relative to that of the murine sarcoma viruses; it varies from 8.2 to 8.8 kilobases. Both the integrated and the proviral DNA sequences of the RHHV and WR-RaLV have been isolated and purified from their respective host cells using a combination of chemical extractions, $CsCl_2$ density gradient isopycnic centrifugation and Reverse Phase V(RPC-V) high pressure liquid chromatography of the restricted DNA by EcoRl endonuclease. The viral genomic DNA was then successfully ligated onto the pBR 322 DNA at the restriction site of endonuclease BamHl and molecularly cloned in E. coli RRI (EK-1) host. The choice of the BamHl site for cloning was a correct one since both the pBR 322 circular DNA and the proviral RaLV DNA each had one single BamHl site. However we later found that both

the Eco R1 and Bam H1 endonucleases wobbled and also created "sticky" staggered ends (diagram) permitting the ligation of both ends possibly with one base mismatching. Upon screening the resultant



clones by drug senstivity tests against ampicillin and tetracycline, we found that out of 5,745 clones of ampicillin resistant clones 14 were sensitive to tetracycline treatment indicating that the RaLV DNA fragment has been successfully inserted into the plasmid pBR 322 DNA at the Bam H1 site which is situated in the tetracycline resistancy locus. Using the "mini-prep" screening technique only three out of the 14 clones showed positive hybridization with the ^{32}P-labeled viral cDNA generated in an endogenous reverse transcriptase reaction, although all showed positive hybridization with ^{32}P-labeled nicked translated pBR 322 DNA. Molecular cloning of as big a DNA fragment as this 8.8 kbp RaLV DNA via pBR 322 DNA vector therefore is of low efficiency. Subsequent nucleic acid analysis of the recombinant DNA of these three clones however showed that the total length of the RaLV genomic DNA had been successfully cloned and that faithful transcript was synthesized in the E. coli RRI cells.

Likewise the 6.4-7.0 kbp fragment of the KSV DNA was ligated onto the pBR 322 plasmid DNA at the BamH1 site and cloned in E. coli RRI cells. Out of 3820 ampicillin resistant clones, 11 were found sensitive to tetracycline. Positive hybridization at varying degrees with respect to the different recombinant clones was obtained. DNA of these recombinants all showed both circular and linear forms. Detailed analysis of these recombinant DNAs has yet to be done.

2. Restriction endonuclease mapping of the cloned viral DNA sequences

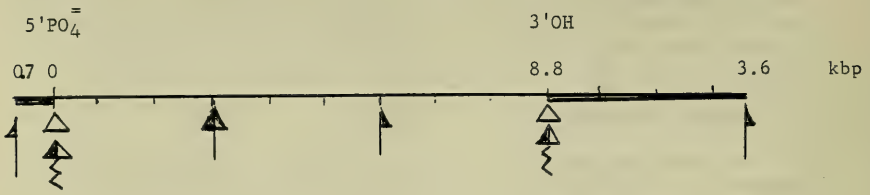
Recombinant clone 8/32 yielded both circular and linear forms of DNA. The linear forms measured at 8.0-8.4 and 13.2 kilobase pair (kbp) in 1.0% agarose gel electrophoresis. When the recombinant DNA was treated with limiting digestion with Bam H1, the circular form was greatly reduced and significant increments in the concentrations of 13.2 kbp band and 8.0-8.4 kbp band were observed.

In addition, a band migrating at 4.3 kbp coincident with the pBR 322 DNA appeared suggesting the cleavage of the pBR 322 DNA from the RaLV DNA at the site of ligation. Extensive digestion (overnight) by BamHl resulted in appearances of 8.0-8.4, 6.5, 3.0 kbp bands, a dense band at the 3.5-3.7 kbp area and a trace of 4.3 kbp band. Thus the BamHl site is located at a point 3.0 kbp proximal to the plasmid DNA which is shown in Figure A.

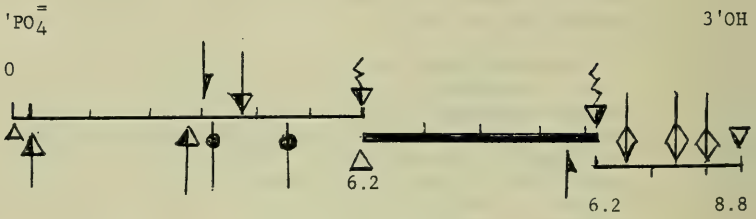
Secondary digestion of the 6.5 and 3.0 kbp fragments by Pst endonuclease resulted in the cleavage of the 6.5 kbp fragment into 3.0 and 3.7 kbp fragments but left 3.0 kbp fragment untouched. This placed the Pst site at 3.7 kbp from the distal end. The placement of these restriction enzyme sites were defined by the γ-^{32}P labeling of the 5' end of the recombinant molecule as described below.

In order to define more specifically the restriction endonuclease sites with respect to the 5'PO_4 end we chose to label the 5' end of the recombinant DNA after limited digestion with Eco Rl by the reverse reaction of the polynucleotide kinase reaction originally described by Maxam and Gilbert using an extremely high specific activity of γ^{32}P-ATP (5700 Ci/mmole). We were able to open the recombinant DNA by a single Eco Rl hit. The 12.8-13.2 kbp fragment was labeled weakly at the 5'PO_4 end. Further digestion with a second hit of Eco Rl rendered the recombinant DNA into an intensely labeled 2.6 kbp fragment, strongly labeled 6.2 kbp and 4.36 kbp fragments, moderately labeled 8.8 kbp fragment which in an extensive digestion further broke down to a labeled 6.2 kbp and an unlabeled 2.6 fragment indicating that the 2.6 kbp fragment should be oriented at the 3'OH end; this was confirmed in subsequent 3' end labeling experiment. Secondary digestion with Kpn on the isolated 5' labeled 6.2 kbp fragments resulted in cleavage of the 6.2 kbp fragments to labeled 5.2 and 3.6 kbp fragments and unlabeled 2.6 and 1.4 kbp fragments. The two Kpn sites could then be assigned to 3.6 kbp and 5.2 kbp proximal to the 5'PO_4 end (Figure B). These two fragments, 5.2 kbp and 3.6 kbp, were further cleaved by Pst digestion into radioactive 4.2 kbp and 3.0 kbp bands respectively, releasing unlabeled 1.0 kbp and 0.6 kbp bands. This placed the two Pst sites inside the 5.2 kbp fragment with one situating between the Kpn sites and the other at 3.0 kbp proximal to the 5'PO_4 terminus. Isolated 2.6 kbp was not digested by Kpn but was cleaved by Taq endonuclease into strongly labeled 2.0, 1.7 and 0.55 kbp fragments releasing unlabeled 0.50, 0.9 and 2.05 kbp fragments. Thus three Taq sites were assigned to the 2.6 kbp fragments as shown in Figure B. Confirmation of these restriction endonuclease sites was obtained in similar experiments with DNA fragments labeled at the 3' terminus with T_4 DNA polymerase I (Dimer) that catalyzes only 3' staggered ends such as that generated by EcoRl (Maxam and Gilbert). The 3' terminus was further confirmed by hybridization experiments with the 3' labeled 2.6 kbp fragment and isolated restricted DNA fragments. Other restriction endonucleases that were found ineffective are Bgl II, Hind II and Hind III. A summary of the restriction enzyme map of the molecularly cloned RaLV DNA is presented in Figure C.

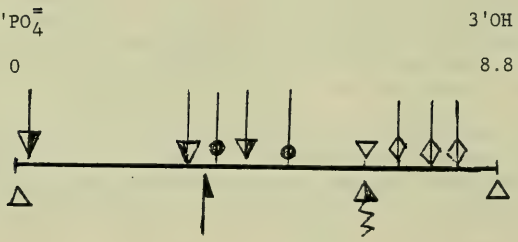
(A)



(B)



(C)



Restriction Endonuclease Map of Cloned RaLV DNA

Restriction Endonuclease	Sites on RaLV DNA
Eco R1	1
Bam H1	1
Wobbling Bam H1	1
Pst	3
Kpn	2
Taq	3
Bgl II	0
Hind II	0
Hind III	0

3. Functional organization of the molecularly cloned RaLV DNA sequence

Molecularly cloned RaLV DNA fragments isolated by agarose gel electrophoresis were blot-transferred to nitrocellulose filter (Southern Technique) and the minute band of DNA stained with ethidium bromide on the nitrocellulose filter was localized by UV light. It was cut out, dried at 80°C under vacuum for 2 hrs and used to select for specific RaLV mRNA by RNA-DNA hybridization in 5X SSC, 50% formamide, 37° for 40 hrs. The tiny filter was washed repeatedly and then the hybridized mRNA was released by elution with sterile distilled water at 85°C for 10 minutes. The mRNA thus obtained was used in cell free protein synthesis in a rabbit reticulocyte lysate system using ^{35}S-methionine as label. The ^{35}S-labeled proteins were immunoprecipitated with anti-RaLV serum and analyzed in SDS-PAGE electrophoresis. Results indicated that the 8.8 kbp fragment coded for: 1) P85, the precursor for the structural protein (2) P70, most probably the gag protein since it is heavily labeled and (3) P29, the rat tumor virus transformation protein (Scolnick). These were compared with ^{35}S-labeled KSV(RHHV) proteins and no difference was observed. Other bands of labeled proteins observed at minor concentration were P115, the polyprotein, P65, P55, P45 and P12, a marker protein for RaLV. Codon for the P29 RaLV transformation protein seems to be located within the distal 3.5-3.7 kbp fragment overlapping the 2.6 kbp fragment. More precise experiments are needed to pin-point the codon locus.

4. IAP specific DNA sequence in DNA of human chondrosarcoma carried in nude mouse

In collaboration with Drs. J. Costa and N. Wivel, IAP cDNA was prepared by the endogenous reverse transcriptase reaction in the presence of Mn^{++}, using ^{3}H-deoxynucleotides as labels. IAP specific ^{3}H-cDNA was then used to hybridize with sheared DNA preparation of human chondrosarcoma carried in nude mouse versus sheared DNA from nude mouse liver and other tissues. By the Wetmur-Davidson plot it was found that 3-4 and 8-10 copies of IAP specific DNA sequence existed in human chondrosarcoma DNA dependent on the preparation of tumor versus a background of 0-1 copy in control nude mouse tissues. This observation correlated with the appearance of IAP in 15% of the human chondrosarcoma cells and the presence of human H-2. Further confirmation of these studies by karyotype analysis is currently undertaken. If it indeed turns out to be human chondrosarcoma cells that carried the IAP specific DNA sequence in vivo culturing of the tumor cells in nude mouse served as an activation mechanism to evoke the expression of IAP.

Relationship of Research to National Cancer Plan:

Project Area - 10

Approach Elements - 2

Approach - 4

Publications

Yang, S.S., Yeh, L-S, L., Taub, J., Miller, N., and Gardner, M.: Integrated and proviral DNA sequences specific for wild rat tumor virus and for rat hepatoma helper virus in various laboratory and wild rat tumor cells: A restriction enzyme analysis. In Essex, M., Todaro, G., and Van Hausen (eds.) Cold Spring Harbor Conferences on Cell Proliferation: Viruses in Naturally Occurring Neoplasm, Vol. 7, December 1980, pp. 1083-1092.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 05550-12 LCBGY

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Role of Retroviruses in Oncogenesis and Tumor Immunogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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3.50	2.50	1.50

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of this project is to investigate the role of type C retroviruses as an etiologic agent and a vector of genetic information for neoplasia, and the immunologic responses of the host to tumor associated antigens.

The topics of current interest are: 1) Further study of the role of cloned B-tropic viruses (11A-MuLV) in transmitting transplantation immunity against Meth A tumor. 2) Characterization of newer isolates of xenotropic, dual-tropic, and amphotropic viruses by competitive radioimmunoassays. 3) Further studies on the interferon and natural killer (NK) activity associated with transplantable and spontaneous reticulum cell neoplasm (RCN) of SJL/J mice. 4) Immuno-potentiating effect of a glucan (Lentinan), and 5) Effects of interferon and tumor promoter on the NK-sensitivity of trophoblast cells.

Project Descriptions:

I. Further study of the role of cloned B-tropic virus (11A-MuLV) in transmitting transplantation immunity against Meth A tumor.

Two SC-1 cell clones (#36 and #46), which were infected with 11A-MuLV at a high m.o.i. and were found to be able to induce transplantation immunity against Meth A cell challenge in mice were further studied. The B-tropic virus being produced by these cells may or may not be a mixture of an immunogenicity-competent but replication-defective virus (I+R-) and an immunogenicity-noncompetent but replication-nondefective (I-R+) virus. By limit dilution procedures (3 times) the viruses were cloned from these SC-1 cells and injected into BALB/c mice in the form of newly infected SC-1 cells to test for their immunogenicity against Meth A tumor challenge. The results indicated no transplantation immunity being transmitted, suggesting that the virus clones isolated were of I-R+ type since they are the majority of viruses produced by #36 and #46 SC-1 cell clones. This experiment proved again that the I+R- type of virus is a minority and probably requires I-R+ virus as a helper for replication.

BALB/c 3T3 cells (clone pf7) were also infected with cloned or uncloned 11A-MuLV and Stansly-MuLV and used for immunization of BALB/c mice. This experiment was done in the hope of obtaining a more marked immunologic stimulation because the syngeneic BALB/3T3 cells would be able to survive for a longer time than allogeneic SC-1 cells would in BALB/c mice. Preliminary results indicated that those cells replicating cloned or uncloned 11A-MuLV gave a significant protection in BALB/c mice against Meth A cell challenge but those replicating Stansly-MuLV did not. The significance of these findings will be further investigated.

The question of the origin of 11A-MuLV was investigated because it is unusual for BALB/3T3 cells from which the 11A cels were derived to produce such a B-tropic virus. The possibility that it has been derived from the activated endogenous MuLV of BALB/3T3 cells was considered. However, competitive radioimmunoassays performed with this virus against ^{125}I-AKR-MuLV p12 and ^{125}I-Moloney-MuLV p12 versus their specific antiserum indicated that 11A-MuLV is more closely related to Moloney-MuLV than to AKR-MuLV. Since p12 is a type-specific gag gene product, the presence of Moloney-MuLV specific p12 in the 11A-MuLV would be considered as evidence for recombinational events between Moloney-MSV gag gene and an unknown endogenous virus. Further study is necessary for elucidation of this problem.

II. Further characterization of xenotropic viruses (isolated from SJL/J mice and Abelson tumor of BALB/c mice), a dual-tropic virus (isolated from Abelson tumor)and amphotropic viruses (isolated from RBL-5 and YAC tumors) by competitive radioimmunoassays.

The type-specific p12 competitive assay results for SJL-MEF-X-MuLV and SJL-RCN-X-MuLV indicated that these viruses were more closely related to BALB:virus-2 than to NZB-X-MuLV or AT 124-X-MuLV. This result was consistent with the fact that SJL/J as well as BALB/c mice produce both ecotropic and xenotropic viruses while NZB and NIH Swiss mice produce only xenotropic viruses (NZB- and AT 124-MuLV respectively). Abelson-X-MuLV is also more related to BALB:virus-2 than to NZB-X-MuLV, but it has its own uniqueness as shown by p12 assays. The dual-tropic NBX virus isolated from Abelson tumor is a recombinant between Moloney-MuLV and X-MuLV as indicated by p12 and gp70 competitive radioimmunoassays, but it acquired a unique gp70 antigen, the origin (parent virus) of which is unknown at the present time.

Competitive radioimmunoassays with new isolates of amphotropic viruses showed that R5NX (from RBL-5 cells) has p12 which is related to AKR p12 while YACNX has no such cross-reacting antigen. This fact is in accord with the tryptic digest map reported previously. Further work is being done to characterize these and other newer isolates.

III. Further studies on the interferon and natural killer (NK) activity associated with transplantable and spontaneous reticulum cell neoplasms (RCN) of SJL/J mice.

Recent investigations revealed the presence of immune interferon (IFN-γ) (acid-labile) in the RCN-homogenate. Intraperitoneal injection of RCN into SJL/J mice induces a transient production of acid-stable interferon detectable in the serum of mice 1 day after injection, but the IFN-γ appears in the lymphoid organs only when the RCN has grown to a certain extent. The increase in NK activity in these RCN preparati;ons may be accounted for by the action of IFN-γ, triggering activation of pre-NK cells to become mature NK cells as well as enhancing the killing efficiency of pre-existing NK cells. The mechanism by which IFN-γ is induced in the RCN-carrying organs is not clear.

In contrast to transplantable RCN, the spontaneously arising, primary RCN in the spleen and lymph nodes of older SJL/J mice display generally weaker NK activity and different range of target cells. Mice bearing transplantable RCN showed a high NK activity against RBL-3, EL4G$^-$, and YAC tumor cells but no activity against a syngeneic lymphocytic tumor, D2, whereas those bearing spontaneous RCN showed a high NK activity against EL4G$^-$ and/or YAC tumor cells, low, if any, against RBL-3 cells, and often high against D2 cells. These activities could not be abolished by removal of macrophages and nylon wool-adherent cells or by treatment with anti-Thy 1.2 serum plus complement.

These observations may suggest that different subpopulations of NK (or pre-NK) cells are being activated during primary development and subsequent transplant passage, each subpopulation representing a clone of NK cells with different repertoire of target antigen-recognition sites (or receptors) on cell surface. Since the primary RCN is pleomorphic, and as the subsequent transplant passages tend to change the histology to monomorphism with reticulum cells as the dominant feature, it is possible that there are changes in populations of putative suppressor cells or other regulatory cells in the RCN site, which may be reflected to changes in populations of activated NK cells. Further investigation is necessary to elucidate the role of interferon and the significance of these observations in terms of immunologic surveillance and evolution of RCN.

IV. Immunopotentiating effect of a glucan (Lentinan).

The present investigation was undertaken to explore the possible antiviral and antitumor effects of lentinan, using the following 3 murine models: 1) vesicular stomatitis virus (VSV)-induced encephalitis, 2) transplantable, allogeneic trophoblast tumors and 3) Abelson virus-induced tumors. The effect of lentinan treatment on NK activity of mice was also examined as one of the possible mechanisms by which lentinan exerts the observed beneficial effects.

Daily injections of lentinan in BALB/c or athymic nude (nu/nu) mice afforded a slight protection against encephalitis caused by vesicular stomatitis virus intranasal infection. However, when (BALB/c x C57BL/6)F_1 mice which are relatively resistant to VSV infection were used, a significant protection was achieved. Pretreatment in addition to post-infection treatment was more effective than postinfection treatment alone. Lentinan treatment also markedly improved the survival of CF-1 mice transplanted with allogeneic trophoblasts which produced ascitic tumors. Despite the absence of H-2 antigen in these cells, the lentinan-treated host appears to exhibit significant resistance, the mechanisms of which is unknown.

Although lentinan post-transplantation treatment did neither reduce tumor incidence nor tumor volume in normal BALB/c mice transplanted with syngeneic Abelson tumor, pretreatment with lentinan for 10 days prior to tumor challenge provided a moderate degree of protection. Lentinan treatment also potentiated the immunizing capacity of small doses of Abelson virus resulting in significant reduction in the size of tumor developing after Abelson tumor challenge. Abelson virus-infected BALB/c nu/+ mice when treated with lentinan showed a higher titer of transforming virus indicating the possibility of activation by lentinan of target cells for Abelson virus transformation.

The natural killer (NK) activity of nude mice was slightly enhanced when the mice were treated with lentinan for 1 day. However, when the lentinan treatment was restarted after a resting period of 4 days following a course of 3-day treatment, there was a boosting

effect on enhancement of NK activity, indicating the importance of finding an optimal condition for boosting NK activity. As NK target cells, trophoblast cells were found to be moderately sensitive, suggesting a possible role for the NK activity of lentinan-treated mice in their resistance against trophoblastic tumors.

V. Effects of Interferon and Tumor Promoter, 12-O-Tetradecanoyl-phorbol-13-acetate, on the sensitivity of trophoblast cells.

It has been postulated that during pregnancy the maternal immunologic mechanism would be exposed to major or minor histocompatibility antigens of the conceptus. However, the placenta containing trophoblast cells which lack expression of major histocompatibility antigen can serve to provide a barrier against afferent and efferent mechanisms of maternal immunologic response against the fetus which bears paternal antigen foreign to the mother in outbred mammalian species.

We have established trophoblast cell culture lines from murine placenta and demonstrated their lack of expression of H-2 antigen, and their ability to grow as carcinomas in different strains of mouse across histocompatibility barriers (Log et al. 1981). The differentiation status of trophoblast cells are somewhat similar to that of embryonal carcinoma cells in that they are H-2(-), resistant to type C retrovirus infection and SV40 T antigen formation, and insensitive to interferon in terms of protection against vesicular stomatitis virus (VSV) replication. Since cytotoxic T cells generally fail to kill embryonal carcinoma cells, whereas NK cells are able to kill these cells (Stern et al. 1980) as well as normal thymocytes and certain stem cells of the bone marrow, it is considered a strong possibility that NK cells would provide an alternative to T cells for cytotoxic cell-mediated immunity against placental trophoblast cells.

^{125}IUdR-labeled trophoblast cells derived from BALB/c, C57BL/6, SJL/J, (BALB/c x C57BL/6)F_1, and CF-1 (non-inbred) mice were used as targets, and spleen cells obtained from NIH Swiss nu/nu mice were used as effectors. A moderate degree of cytotoxicity was observed with these targets which were generally comparable or slightly lower in sensitivity than that of YAC cells. Removal of macrophages and nylon wool-adhering cells from the effector cell preparation did not diminish the cytotoxicity. The nature of NK cell-binding surface membrane structure of trophoblast cells which lack expression of H-2 and type C retroviral antigens is currently under investigation.

Since interferon is known not only to increase both the efficiency and the number of cytotoxic NK cells, but also exert a protective effect on certain target cells against NK cytotoxicity (Trinchieri et al. 1978; Hansson et al. 1980), the effect of interferon on trophoblast cells was tested for their sensitivity to NK cells which had been treated or untreated with interferon.

It was found that interferon pretreatment of trophoblast cells resulted in a significant decrease in their sensitivity to NK cell cytotoxicity. Treatment of effector cells with interferon enhanced the cytotoxicity against trophoblast cells which more than counteracted the lowered sensitivity of interferon-treated target cells.

These effects of interferon were detected by use of both crude and purified murine interferon (IFN-β) and could be abolished by anti-interferon serum.

It is of interest to note that interferon treatment of trophoblast cells did not give a significant protection againt VSV infection although similar treatment of L cells or fibroblasts readily gave a profound protection. Treatment with high concentration of interferon (100 units) did not protect trophoblast cells against VSV or encephalomyocarditis virus. However, treatment of trophoblast cells with moderate dose (20 units) of interferon was capable of reducing their sensitivity to NK cell cytotoxicity. Thus, a dissociation between virus and NK susceptiblity could be achieved with trophoblast cells interacting with interferon. The molecular mechanism of this phenomenon is currently under investigation.

Fowler et al. (1980) reported the presence of high titers of interferon in murine placentas although the mechanism of induction and accumulation in placenta of interferon was not clear. The biologic significance of interferon in placenta should be considered in the light of the above findings.

Furthermore, an attempt was made to modify NK sensitivity of trophoblast cells (treated or untreated with interferon) by treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA), which is the most potent tumor promoting agent among phorbol esters and capable of exerting pleiotropic effects on cell differentiation and proliferation (Weinstein and Wigler, 1977; Diamond et al. 1978; Colletta et al. 1980).

Culture of trophoblast cells for 1-2 weeks in the presence of 1.6×10^{-7} M TPA in the medium (RPMI 1640 supplemented with 20% fetal bovine serum and 50 μM mercaptoethanol) resulted in a marked decrease in the sensitivity of these cells to NK cytotoxicity. However, the ability of these TPA-treated cells to compete against labeled target cells for NK cytotoxicity was not diminished, indicating that the resistance to NK cytotoxicity is not due to the lack of target membrane antigen recognized by NK cells but is probably due to the resistance of those cells to lytic effector mechanism. Paradoxically, treatment of these TPA-treated cells with interferon readily elevated their NK sensitivity to the original level before TPA-treatment. The exact biochemical mechanisms for these changes are being studied in the light of mechanisms of differentiation or dedifferentiation.

In any event, the microenvironment in which the trophoblast cells of placenta reside may be considered to be the site where external carcinogen or tumor promoting agent may come in and interact with the locally produced or accumulated interferon to exert effects on

maternal NK cells or other immune mechanisms as well as on the trophoblast cells as target to these immunological forces. The importance of such immune surveillance mechanism, if it exists in humans as well as mice, may be visualized (although conjectural), when failure in this homeostatic system were to occur, resulting in local or systemic dissemination and multiplication of trophoblast cells causing trophoblastic diseases such as choriocarcinoma.

Publication:

Log, T., Chang, K.S.S., and Hsu, Y.C.: Carcinomas induced by cell lines cultivated from normal mouse placentas. Int. J. Cancer 27: 365-372, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05552-12 LCBGY

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mammalian Cellular Genetics

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4.7	3.5	2.5

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to analyze and develop new and difficult cell systems in culture. We have developed and are attempting to exploit applications of normal rat thyroid cell cultures. These cells are hormone dependent. They synthesize and secrete a very large protein product, thyroglobulin. They concentrate iodide 100-fold from the medium. They offer a unique opportunity to study secretion, ion uptake and cAMP response. These are being studied in our lab and in other labs, however, our approach is primarily to use electrophysiological techniques. We are attempting to study long term regulation of membrane potential and its relationship to secretion and hormone levels. We are also studying neurons and neuroblasts in cell culture. There are too few mammalian cell systems where "blast" cells can be observed in transition to mature, differentiated cells. We have tried this in nerve cells using cellular hybridization and cellular transformation (with ts SV40 viruses) and by using little known cell systems in which blast cells persist throughout life (olfactory epithelium). We are especially interested in the development of electrophysiological competence and in the development of chemical identity (specificity).

I. Introduction

During the past year we have experienced a shift in emphasis of the main thrust of our work. We are moving away from concentration on the manipulation of cellular genes toward the development of the new and much needed cellular systems themselves. This has come about for two reasons: 1) the field of transfection, cellular hybridization, and cybridization have now become crowded (our lab was one of the pioneering groups in this field and now they seem to have caught on), 2) It now seems clear that there are greater urgencies in cell biology. It appears that either the techniques have become rather uninformative or they have become the province of mega-labs demanding itterations of the same procedures in order to yield the final goal of this work: a DNA sequence. In either case, the best use of a small group like ours would seem to be to remain at the uncrowded cutting edge of cell biology where the development of important new cell systems is both our forte and much needed by the field as a whole. Meanwhile, our expertise in hybridization, cybridization, transfection, and microinjection will be kept alive by small (mostly intramural) collaborations and thus we shall remain ready to pounce when and if these techniques are demanded by our own projects.

II. Microinjection experiments

A. Cloning of malaria

A major breakthrough occurred on May 15, 1981 when we found that 5 out of 5 monkeys had been successfully injected with malaria, each one by a single, isolated parasitized red blood cell from an infected donor monkey. The first of these monkeys have proven to be SECA positive, indicating that splenectomy and not a possible defect in cloned parasites caused the strain that came from our sole previous success to be specific antigen negative. The secret of this success turned out to be a combination of 1) choice of healthy infected cells, 2) rapid transfer of the infected cell to 37°C and 3) prompt addition of an excess of red cells from the particular monkey to be inoculated. Whilst incubating for 40-60 minutes the single, infected cell matured and released parasites that immediately infected the soon to be "host" monkey's own red cells. The take rate is very high when all of these factors are combined. These five successful clones mean that our 2 year effort was finally successful and that a new technology has been born.

B. Assay of mitogenic effects of insulin after microinjection into human fibroblasts

In collaboration with Drs. R. Kahn and G. King (A,D) we have undertaken to see if we can microinject insulin into human fibroblasts and then see whether the suspected mitogenic effect of naturally internalized insulin is observed. Individual human fibroblasts will

be located and marked using an objective marker (diamond scriber) and then the cells within the marked area will be microinjected with highly concentrated solutions of purified insulin. The cultures will then be incubated in 3-HTdR and prepared for autoradiography. Surrounding cells (outside of the marked region) will serve as one kind of control. High frequency of TdR incorporation will be provisionally interpreted as conformation of this role for internalized insulin. We have contemplated doing this experiment for some years but have not done so because of the fear that insulin leading from the pipet might interfere with the specificity of the response. However, in order for exogenously supplied insulin to have any mitogenic effect it must be present continuously for 3 or more hours. Therefore, if the experiments are done expiditiously then the result should clear. The preliminary work has been accomplished and the injections are about to begin.

III. Culture of differentiated cells

A. Thyroid cells

Our Fisher Rat Thyroid cells grown in low or no serum (FRTL) have continued to provide a powerful resource; they are the first and possibly only example of a karyotypically normal, long term cell strain that has retained complex differentiated functions including biosynthesis of physiological amounts of thyroglobulin (TG), concentration of 100-fold or more of iodide, and apparently normal response to the hormone thyrotropin (TSH). We have sent these cells out to laboratories around the world and they have provided the basis for numerous studies involving hormone mechanisms - especially the response of the cyclic AMP system to TSH stimulation.

1. Human thyroid cell strains:

However useful these rat cells may be there is always a premium paid (especially by the physicians) on having a human cell strain available. After Dr. Ambesi-Impiombato and I succeeded in developing strategies that were successful in getting rat cell strains we thought that surely there would be no difficulty in getting human cell strains in the same way. That was not to be. Human cells have been supplied by Dr. William Valenti from surgical procedures on diseased thyroids which necessitated removal of small amounts of apparently normal tissue as well as from the "instant autopsy" program of the University of Maryland. In collaboration with Drs. Ambesi-Impiombato, Valenti, and L. Kohn we have made many attempts to culture human thyroid cells without success. Recently, during his visit from Naples, Dr. Ambesi has succeeded in overcoming the "human thyroid program". The same media and hormone supplements used for the rat cell lines are used but no serum (fetal calf, calf, horse, human, etc.) can be tolerated by the human cells at any time. Furthermore, they require self conditioned medium for long term survival. It has been known for some time that certain tumor cells and transformed cells may produce growth simulating factors in the culture medium and recent evidence

suggests that even normal cells may produce growth factors such as insulin (albeit in very small quantities). Apparently the human thyroid cells require a factor that they themselves synthesize. This sort of "autocrine" phenomenon may be related to the chelones and other similar growth regulating substances that are vital to maintaining the integrity of groups of cells in tissues and may very well have important implications for the future understanding of the cancer problem. At any rate, the availability of human cell strains with thyroid function appears near reality. Such cell strains, if they are like the rat cell lines will prove important developments. Whenever we learn something about how to grow normal, differentiated cells in culture we are getting closer to understanding how these cells are integrated into tissues and organs and are therefore, closer to understanding how those mechanisms can go astray.

2. Continued work with the FRTL strain

a) While Dr. Ambesi was visiting our laboratory we started an effort to produce variants (mutants) of the FRTL cell strain that no longer produced TG. Such nonproducers are not seen in our normal cultures when they are screened with FITC-anti TG. Small clumps of TG are always seen on the surface of every FRTL cell (they are not seen on FRT (a non-functional epithelial cell from the thyroid that we believe has secondarily lost the ability to synthesize TG). We determined that the best way to screen for TG-nonproducers among mutagenized FRTLs would be to adapt the agar + medium + antiserum overlay technique developed by Coffino, Laskof and Scharff for similar screening for IgG-nonproducers. This procedure worked very well indeed. We produced rabbit anti-rat-TG and incorporated it in agarose + 6H medium and overlayed the clones grown from populations of FRTL that had been exposed to standard mutagens (e.g., EMS). After 24-48 hours a precipitate could be plainly seen over the majority of colonies and none was seen over a very few (estimated one putative non-producer per 2000 colonies). Some colonies seemed exceptionally active. Both types of colony are being isolated for further study. Using techniques like subselection and/or further mutagenesis it should be possible to produce a wide variety of mutants deficient and superficient in different portions of the system that regulates TG production. To date, the most significant part of these results is the development of a successful screening technique.

b) Dr. Nelson Sinback has continued his work on the electrophysiology of the FRTL cells. Dr. Sinback has studied the ionic mechanism of thyroid cell secretion in response to catecholamines. He has recorded from single cells and iontophoresed norepinepherine onto the external cell membrane (norepinepherine causes depolarization of the membrane potential). He has perfused medium with altered Na+, Ca++, Cl-, or K+ concentration outside the cell to determine ions responsible for response to conductance to Cl- and Na+. Both Cl- and Na+ conductances are increased by release of intracellular Ca++. Injection of EGTA from a second

intracellular electrode abolished depolarization to norepinepherine depolarization.

Perhaps the most exciting development of the past year was our (Dr. Sinback's) discovery of slow oscillations in thyroid cell membrane potential. These oscillations of 10-30 mV amplitude and 5-10 min period represent a hitherto completely unknown and unobserved property of secretory cells. Because of the thyroid's strategic role in regulation of metabolism in virtually all other cells, the impression of such a cyclic clocklike influence on the body may be a profound one indeed. As yet, however, we have not linked the periodic membrane potential changes with secretion. If that is done then there will be profound medical implications as well as implications to theoretical biology of this phenomenon.

Some details that are now emerging: Oscillations occur spontaneously in some cells and are induced by injecting H+ ions in others. Also, they are recorded in greater fraction of cells in low TSH medium. Dr. Sinback has not been able to record any change (increase or decrease) in ion conductance associated with the oscillations. Thus, it may be some electrogenic ion exchange, for instance Na^+, H+ which is regulated by internal H+ concentration. This implies some physiological role, unknown but perhaps TG secretion is regulated by these oscillations. The results predict that CO_2 saturated medium would increase TG secretion. In the near future, Dr. Sinback will try to measure the intracellular pH to see if there is a correlation with amplitude or period; also, to see if intracellular pH of low TSH grown cells is different from those grown in the usual concentration (2 mU/ml). Also, he will try to measure single Cl- ion channel conductance turned on by norepinepherine using an extracellular patch electrode attached to a 1 um diameter piece of external cell membrane. If he can do this he will then be able to determine more about the Ca++ regulation of channel open time and conductance.

c) In the past year Dr. Magnusson has continued his studies of iodide metabolism in the thyroid follicular cell strain FRTL. These studies have led to the development of a subcellular fraction of vesicles capable of concentrating tracer iodide up to 80-fold in relation to the incubation media. Dr. Magnusson has developed the assays and computer programs necessary for the quantitative study of these fluxes. The vesicle system, unlike the whole cell, is not inhibited by ouabain or oligomycin, indicating the possibility that the iodide flux is being driven by an energetic state which has been "trapped" at the time of preparation of the vesicles. The stimulation of the iodide flux by bicarbonate, along with the inhibition of this flux by the proton ionophore CCCP may indicate a role for protons in the mechanism of both subcellular iodide flux and whole cell iodide uptake.

Dr. Magnusson has also developed several new thyroid cell lines and has followed the synthesis of thyroid hormones (T3 and T4) by these cell lines as a function of time in culture. Preliminary data show a decrease in this synthesis as the cells continue to be grown in monolayer culture. This may help to explain the loss of T3 and T4 synthesis by our older chromosomally normal cell strains.

B. Neurons and neuroblasts

1. CBSVIA

As noted in last year's annual report (and manuscript in preparation) we have attempted to study the cell type that has proven the most refractory to cell culture methodology, the uncommitted embryonic or "blast" cell by using a temperature sensitive SV40 mutant and transforming these cells. Cell lines have been produced by infecting suspensions of cells from the embryonic cerebellum. It was hoped that such cell lines could be propagated at 33°C and caused to return to a more normal state - perhaps to differentiate - at the non-permissive temperature of 40°C. We found that most commonly an intermediate result is attained. The neuroblasts continued to propagate (and to produce the "T" antigen) at 33°C but that as the cultures became crowded, cells that looked like differentiated neurons would appear. It developed that these neuron-like cells had concomitantly lost the "T" antigen (by FITC-anti-T fluorescent staining). These differentiated cells apparently fail to accumulate detectable quantities of "T" in their nuclei in spite of the fact that the culture is kept at the permissive temperature of 33°. They do not divide; they do not resynthesize T antigen. If the cultures are shifted up to 40°, all cells become T-negative in a few days but no new "neuron-like" cells appear. As long as they are incubated at 40° the neuron-like cells that have already differentiated at 33° may continue to improve (as measured by accumulation of neuron-specific enolase, 14-3-2, and/or decreased resting membrane potentials). But new neurons probably do not form. We are still trying to find out what happens to cells shifted back down to 33°: do they resume T-antigen accumulation? Do they divide? Because not all cells differentiate it is difficult to tell whether those that do divide when shifted back to 33° are ever those that were differentiated at 40°. Similarly it is hard to tell if the cells that were differentiated at 33° ever resume division.

It has proven possible to get nearly pure populations of cells in both the neuroblast-like state as well as in the differentiated state. Cultures growing at 33° if fed FUdR in thymidine free medium become progressively enriched (to over 95%) in the differentiated form of the cells. The dividing neuroblast-like cells die after incorporation of FUdR. If cultures of dividing neuroblast-like cells are maintained in log phase by rapid passage at low densities relatively fewer of the differentiated cells appear and if either

the 0.5% calf serum or a crude brain extract is left out of the medium no differentiated cells are formed. Thus highly purified populations of the blast cell and of the differentiated form are available for study. This situation is probably unique among cell systems showing differentiation in vitro. Certainly it is unique for nerve cells.

2. Olfactory epithelium

One of the reasons we chose the cerebellum for the study of neurons and neuroblasts is that there are relatively few different kinds (5 in all) in that region. We wanted simpler systems in which it appeared very likely that we could find both partners in normal synapse formation. We have decided on the olfactory system. Few biologists remember that the sensory element, the sensory neuron in the olfactory epithelium has a half-life of about 15 days in higher mammals. Throughout life the neurons that react specifically with myriad odor bearing chemicals die and are replaced regularly. It may be possible therefore to obtain cultures of the blast cells that proliferate these specific sensory neurons. We cannot find evidence that this cell system has ever been studied in cell culture before. We have initiated cultures from the sensory epithelium in the near term rat embryo (20 days) and have identified the several relevant cell types. We can grow them, clone them and are trying to find ways to regulate their differentiation. To date there are encouraging results. We find that differentiated neurons do appear in our mass cultures throughout several passages which means that the neuroblast is alive and propagating in our culture media.

Simultaneously we are attempting to get cell strains from the olfactory bulb and to transform them with the hope of producing cell strains like CBSVIA from the mitral cell. Such cells are the only known synaptic partner of the sensory neurons in the olfactory epithelium.

One of the special features that make the olfactory neurons intriguing objects for study is the problem of the specificity of the olfactory receptors. Each olfactory neuron must have at least one receptor structure that is capable of reacting with some chemical prosthetic group. The mechanism of that specificity is the interesting question. It is possible that like taste, the number of discrete receptors is low - say 4 or 5. But there are many thousands of odor bearing chemicals that are distinguished. This could be accomplished by designing a system that registers discrete levels on each of the 4 or 5 receptors. Depending on the number of cells bearing a particular receptor that are stimulated (analogous to the binding constant) a discrete signal may be registered on the brain. If there are ten levels of discrimination possible on each of 4 channels that system could, in theory, recognize 10,000 discrete chemicals. In another solution to the same problem the sensory neurons might utilize a variable protein producing mechanism - possibly even the immune system itself in order to generate a large number of unique receptor structures. Either way, different

receptors must be present on these sensory cells. If we can clone the blast cells that produce them we should be able to find the proteins that distinguish them. We shall try by using 2-D gels and hope to pick up a variable spot(s). If that should fail then conventional methods of isolating receptor complexes will have to be tried.

IV. 2-D gel analysis and characterization of cell cultures

We have determined that the power of the 2-D gel systems introduced by O'Farrel may be sufficiently great to make characterization of cell strains much more complete and easier. These gels display nearly all of the cells' soluble protein and may be sufficiently reproducible to provide useful fingerprints of individual cell strains. We are applying this technology to comparisons between blastic phase and differentiated CBSVIA cells (s.v.) and intend to try to use them to characterize different FRTL variants (s.v.). Because the gels are complex (as many as 500-1000 spots) and somewhat subject to artifact their interpretation is difficult. We hope that the difficulty will be relieved by computer analysis. Both DCRT and Carl Merril's group have computer programs that assist in rationalizing 2-D gels. That technology is not yet mature, however, and in its most useful form is at or near the cutting edge of image analysis and computer artificial intelligence. These are areas of our own expertise and we hope to be able to use the computers critically in our attempt to make the gels useful.

In the coming year we intend to make a major effort to develop the 2-D gel technology as a routine adjunct to cell culture work. We would like to determine if ^{125}I or FITC-antibody methods can be used to identify component spots in the gels. We would like to identify known antigens such as neuron-specific enolase and thyroglobulin and to see if this technique could assist in screening monoclonal antibodies produced in response to different cell strains.

If these applications can be developed they will have very far reaching value in the eternal problem of adequate characterization of cell cultures. It may prove possible to standardize cell lines which are notoriously variable by periodic comparison of 2-D gels to bench mark standards. In fact, it is hard to imagine any area of cell culture that will not be aided by this approach.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05553-12 LCBGY

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunoglobulin Structure and Diversity
Characterization of cell membrane proteins

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Immunoglobulin structure and diversity: 1) Amino acid sequence analysis of a series of hybridomas with specificity for β(1,6) galactan is in progress to evaluate structure, function and idiotype. Nucleic acid probes are being developed to characterize the gene structures coding for these proteins. 2) Amino acid sequence analysis of antigen binding variants of a phosphorylcholine binding myeloma has demonstrated that single amino acid substitutions in framework regions as well as complementarity determining regions may alter antigen binding specificity providing a potential model for somatic mutation in the generation of antibody diversity.

II. Cell membrane proteins: Papain fragments have been prepared and purified from the major transplantation antigens obtained from two of our inbred herds of miniature swine. Amino terminal sequences have been obtained for the heavy chains as well as the associated β_2-microglobulin. DNA probes have been used to characterize the genes coding for these antigens by Southern blot analysis and to isolate genomic clones containing transplantation antigen sequences.

PHS-6040
(Rev. 2-81)

I. Immunoglobulin structure and diversity

One of the major ongoing programs in this laboratory is a study of the structure of antibody molecules and the mechanisms by which the diversity in the large immunoglobulin repertoire is generated. We have previously approached this problem by protein sequence analysis of myelomas and hybridomas with defined antigen binding specificity. More recently we have begun to employ the newly developed recombinant DNA technology to examine the structure and organization of genes involved in these particular responses.

a) In our previous report we described the complete amino acid sequences of the κ light chains from 6 myeloma proteins with specificity for β(1,6)galactan containing antigens. It was noted that among these proteins all four J (joining) region segments were used in conjunction with a single κ variable region and that no changes in specificity or affinity were observed in spite of these structural differences. Furthermore, position 96 at the junction of V and J was not encoded in either V or J but was generated by the recombination event and in 5/6 proteins was Ile. To assess the fidelity of this unusual V-J recombination and the diversity among anti-galactan antibodies we have begun amino acid sequence analysis of 11 hybridomas demonstrating the same specificity. Complete sequence analysis of these hybridomas will permit an evaluation of structural diversity, functional diversity, idiotypy and the mechanisms involved in the generation of these molecules. Correspondingly, we have isolated messenger RNA coding for both the light and heavy chains from one of the anti-galactan myelomas. We have constructed cDNA from this message and the cDNA has been cloned in a plasmid vector. Nine kappa and four alpha chain clones have been isolated and are currently being characterized. If any of these clones possess sequences corresponding to their respective V regions they will be used to isolate and characterize the germ line genes coding for these molecules. Thus, a complete description of anti-galactan antibodies from genotype to phenotype will be possible.

b) It is now clear that there exist a large number of immunoglobulin germ line genes and that the respective light and heavy chains are encoded in multiple gene segments. It is not known, however, whether the structural diversity created by recombination of these gene segments as well as potentially subsequent somatic mutation are effective means of generating functional antibody diversity. To determine the effect of a small number of amino acid substitutions as a potential model for somatic mutation we are examining antigen binding variants of the phosphorylcholine (PC) binding myeloma protein S107. An S107 cell line has been adapted to tissue culture and variants with altered antigen binding have been selected. A number of studies have indicated that the alterations in antigen binding are associated with the heavy chain and we have therefore undertaken amino acid

sequence analysis of these chains. Previous determination of the heavy chain structure from two variants revealed a single identical substitution in the J segment of these molecules while the complementarity determining regions were found to be identical. Thus it appears possible that substitutions in the framework portion of the molecule may be able to effect antigen binding. We have now determined the heavy chain structure from an additional molecule which has completely lost the ability to bind antigen and observed a single amino acid substitution in the first complementarity determining region. Based on the known structure of the PC binding site derived from X-ray analysis, the substitution we have observed provides a clear structural basis for alteration in the binding site configuration and subsequent loss of activity. This is the first demonstration that single or few amino acid interchanges which presumably could be generated by somatic mutation, can be effective in altering antigen binding specificity.

We are further using the PC system to examine the evolution of specific germ line structures. A number of hybridomas have been generated in inbred strains expressing allelic forms of the PC heavy chain which we have previously identified. Sequence analysis is being performed on these molecules to assess the distribution and regulation of these alleles in appropriate inbred strains. To further assess the evolution of these alleles we have determined their phenotypic expression in our wild mice colony which represents species and sub-species of the genus Mus both closely related and very distant in evolution from the inbred laboratory strains. We have been able to trace the occurrence of these markers throughout the colony which represents a very long evolutionary period and are now, with the use of molecular probes, beginning to study the evolution of specific genes coding for this response.

II. Characterization of cell membrane proteins

a) In the Annual Report, 1980 we described pertinent features of a large animal transplantation biology model in miniature swine and initial experiments aimed at the isolation of transplantation antigens for chemical and biological experiments. We have now prepared and purified papain fragments of the major transplantation antigens (SLA) from two of our three herds. The SLA heavy chains have been separated from non-covalently associated β_2-microglobulin and NH_2-terminal sequences determined. We have identified the N-terminal 40 amino acids from one haplotype and 36 from the second, as well as 45 from the associated β_2-microglobulin. With the exception of the human, these are the longest transplantation antigen sequences so far determined using non-radioactive (micro-sequencing) techniques. Appropriate comparisons have been made with available sequences from other species.

Since the amount of material required for a complete protein structure would be considerable, we have chosen to use purified antigens for biological experiments and to determine the structure of these proteins through analysis of their corresponding genes.

Using a cDNa probe to human transplantation antigens we have been able to characterize porcine SLA and SLA-like genes by 'Southern' blot analysis. We have also isolated a genomic clone containing SLA sequences by screening of a genomic phage library with the human probe. Experiments are now beginning to characterize and sequence this clone. Using this same procedure we have similarly isolated a genomic mouse H-2 clone which will eventually be used to study the evolution of H-2 genes in our wild mice colony.

In addition to the transplantation antigens the major histocompatibility complex also encodes a series of gene products involved in the regulation of the immune response and cell-cell interaction. These I region gene products have been characterized in our three herds by two-dimensional gel analysis and attempts to purify these molecules are currently underway.

It has previously been suggested that I region molecules (Ia antigens) may be involved in T cell factors or receptors. To examine the potential role of these molecules in T cells we have made a direct comparison of T and B cell Ia antigens by in vitro labeling followed by immune precipitation and two dimensional gel analysis. The Ia patterns from T and B cells were found to be indistinguishable and restricted in number suggesting that they would not be capable of generating significant specificity in biological systems. Studies are continuing to determine whether additional Ia antigens to those observed in common to T and B cells may be present on T cells but not on B cells using antisera prepared against T cell blasts.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05596-12 LCBGY

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Pathogenesis of plasma cell neoplasia: characterization of antigen-binding proteins

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SUMMARY OF WORK (200 words or less - underline keywords)

The pathogenesis of peritoneal plasmacytoma development in BALB/c mice appears to involve a genetically-determined component and a pathological tissue microenvironment that selects preplasmacytoma cells. Identification of genes controlling susceptibility is being approached by making BALB/c congenic strains. Mechanisms involved in the selective proliferation of pre-plasmacytoma cells on oil granuloma is being studied in vivo and in vitro. Characterization of the structure of idiotopes on galactan binding myeloma proteins and epitopes on hen egg white lysozyme is being made with monoclonal antibodies and molecular models. New Vk genetic markers are being characterized. Mammary tumorigenesis in wild mouse populations is being characterized.

PHS-6040
(Rev. 2-81)

Plasmacytomagenesis

Using the LBI-closed colony environment we have been able to carry out plasmacytomagenesis experiments that involve watching mice for one year after the first injection of oil to quantitatively determine plasmacytoma incidence. Success depends upon the freedom from intercurrent infections. We have refined the methods of induction to provide more rapid assays. The standard treatment of three 0.5 ml doses of pristane at days 0, 60 and 120 which gives a 60% yield of plasmacytomas will probably be replaced by a single 1 ml dose. Initial studies have given a 44% incidence by 284 days with a single dose. Another series of experiments have been done with two 0.5 ml doses given at different intervals. A single 0.5 ml dose produces a low incidence of plasmacytomas with 20% or less. When a second dose is given at 30 to 120 days the incidence increases to 40 to 60%. We have now completed three experiments with a day 0-180 2 dose schedule and found that the additive effect can be remembered for 180 days. This result suggests the target cell in plasmacytomagenesis is probably the B-lymphocyte since it would not be expected that a plasma cell would persist for this long in the organism. Thus, plasmacytomagenesis could begin in a cell that does not express the neoplastic trait, and only when the key differentiation step supervenes does the neoplastic trait become expressed. Pristane probably has at least two biological effects on plasmacytomagenesis. First, it may stimulate B-lymphocyte proliferation, and second it creates a more permanent granulomatous microenvironment that is involved in the selection of abnormal cells for growth. Proliferation can activate endogenous type C RNA retroviruses as shown in collaborative experiments with Dr. Janet Hartley, NIAID. Retrovirus activation can lead to the formation of new recombinant virus forms, that are more efficient in re-integration or recombination with host genes.

Arthritis

In addition to inducing plasmacytomas pristane also induces the formation of a chronic arthritis in BALB/c mice. This affects the ankle joints, and feet but can involve other joints. Some mice develop ankylosis. In any given experiment from 10 to 30% of the mice develop arthritis. The interesting feature of this arthritis is the long latent period beginning 4 to 5 months and lasting throughout the time when plasmacytomas develop and lack of evidence of mycoplasma infection. A possible mechanism is an autoimmune process beginning with a destructive tissue change in the peritoneum. The evidence to date indicates the development of arthritis is dependent on the amount of pristane, i.e., 0.5 ml induces a very low incidence 1.0 to 1.5 ml of oil produces relatively high incidence.

We have one clue relating to pathogenesis that is awaitng confirmation. BALB/c Ig congenic strans CB-20 and CAL-20 mice develop a very low incidence of arthritis. This suggests genes linked to the Igh complex on Chr 12 are playing a role in the pathogenesis

of arthritis. Dr. Arthur Anderson of the University of Pennsylvania is studying the pathology of this arthritis by electron microscopy.

Karyotypic analysis of plasmacytomas. In collaboration with Dr. G. Klein of the Karolinska Institute, a karyotypic analysis of primary and early transfer generation plasmacytomas has been carried out using G-banding to identify specific chromosomes. Among 18 tumors analyzed several characteristic translocations have been identified: T6;15, T12;15, deleted 15. Sixteen of the 18 tumors have the T12;15 and del 15 abnormality and 3 have the rcpT6;15. All of the latter are kappa chain producers. In a series of 8 lambda tumors none has rcpT6;15. Since chr. 6 carries the K-chain genes and chr. 12 carries the heavy chain genes, there seems to be some association with Ig-gene complex loci. This is further strengthened by the observation that the translocations (like the allelic exclusion phenomenon) involves only 1 haplotype in a cell and in tetraploid cells only 2 of the 4 chromosomes are involved. The break in chr. 15, however, is consistent throughout and appears to occur in the same band site. Because trisomy of chr. 15 is implicated in T-cell leukemias in the mouse, it is possible that a break in chromosome 15 may have an important biological effect on the differentiation and development of lymphocytes.

Continuing our active collaboration with G. Klein, we are attempting to induce plasmacytomas in mice carrying the T6 translocation. T6 is carried on a CBA background a mouse that is presumably plasmacytoma resistant. The T6 translocation involves chrs. 14 and 15. Thus, if plasmacytomas could be induced in mice that have a normal 15 and a 14;15 translocation, it would be of interest to determine which chromosome develops the T12;15-del 15 translocation. For this purpose we are introgressively backcrossng T14;15 and del 15 (from T6) onto BALB/c and attempting to induce plasmacytomas with pristane. We have also obtained two other translocation stocks for this purpose T10;12 31H from Harwell and the T5;12 from Oak Ridge. These translocations are also on plasmacytoma resistant strains.

Since little is known about chr. 15 in the mouse we are studying the effects of other marker genes and their neighbors located on chr. 15 for their role in plasmacytomagenesis. We are testing bt (belt) on C57BL/Ka which can be assayed at BC_2; caracul (Ca) which probably can also be similarly assayed. The presumption here is that genes controlling susceptibility in BALB/c are located on chr. 15.

We are further pursuing the karyotypic analysis of plasmacytomas with Abelson induced plasmacytomas (ABPcT) and lymphosarcomas (ABLS). S. Ohno of Kanazawa University, Japan has nearly completed this study. ABLS are balanced diploids. Two of five plasmacytomas are either diploid or tetraploid and do not contain translocations. The other 3 have rcp T6;15 but no 12;15. This result suggests ABLV in some way blocks the translocation of chr. 12 to chr. 15 and this permits a greater chance for rcp T6;15 to occur.

Identification of genes confering susceptibility to mineral oil plasmacytomagenesis in BALB/c mice. Most inbred strains of mice are not susceptible to peritoneal plasmacytomagenesis. The remarkable susceptibility of strains BALB/c and NZB suggests specific genes are involved. F1 hybrids of BALB/c and the resistant DBA/2 strains are resistant to plasmacytomagenesis suggesting resistance genes are dominant. We are attempting to identify resistance (R) genes in DBA/2 by backcrossing a number of DBA/2 loci onto the BALB/c genotype, to produce BALB/c congenics carrying R genes.

The aim of this work is to identify the resistance (R) genes, as a first step, and then to test whether the allele of the R gene is a susceptibility gene by linkage association. The ultimate aim is to find the product or the function of the susceptibility gene and show how it leads to neoplastic change in plasma cells.

We are breeding or currently raising test progeny from N6 for: Idh-1 (Chr 1) Fv-1 (Chr 4); Pgm-1 (Chr 5); C (Chr 7); Es-3 (Chr-11); IgC_H (Chr 12); Lyt 1 (Chr 19); Ly-2 (Chr 6); Qa-2 (Chr 17). Isabelle Pawlita has worked out the cytotoxicity tests for the last 3 genes. In collaboration with Janet Hartley, NIAID, we are introducing a DBA/2 gene that is resistant to MCF viruses onto BALB/c that controls resistance to MCF viruses.

We are also preparing congenics carrying 2 loci on the same chromosome so that a larger part of the chromosome can be introduced (Idh-1 and Dip-1) on Chr 1 and (Pgm-1 and β-Gus) on Chr 5.

We have now completed a study of the Pgm-1 (Chr 5); C (Chr 7) IgC_H (Chr 12) and Fr-1 (Chr 4) and have not found resistance genes associated with these markers. Congenics carrying other markers are nearly ready for testing.

To include larger regions of DBA/2 chromosomes, we have at the suggestion of Dr. Eva Eicher of the Jackson Lab, begun making BALB/c congenic carrying x-ray or mutagen induced paracentric inversions. Dr. Thomas Roderick has developed these stocks. The large inversions prevent or reduce crossing over, thus allowing the introduction of chromosome segments that could be as large as 60 or 70%. Many of these inversion stocks were induced in DBA/2 thus, we are able to extend our list to include many new linkage groups including chromosome 15.

A study is being completed on C3H/HeJ and C3H/HeN, both of which are resistant to developing plasmacytomas. This will provide a new genomic source of resistance genes. The LPS-sensitivity gene of C3H/HeN origin has been backcrossed onto BALB/c to N6 and the effect of this gene will be tested this year. The CBA/N x linked gene that controls the development of a subpopulation of B-lymphocytes was backcrossed onto BALB/c An by Dr. Carl Hanson is currently being tested and this stock is resistant to plasmacytomagenesis.

A second approach to finding genes that are associated with susceptibility and resistance is to compare the susceptibility of BALB/c sublines. We are currently submitting for publication a study of BALB/c J and BALB/c Boy. BALB/c J develop only 10-20% plasmacytomas as compared with 60% in BALB/c An and BALB/c An Boy. We are currently examining BALB/c Argonne, and will soon study BALB/c ORNL and BALB/c. The last 2 mentioned substrains were separated from BALB/c An over 46 years ago.

The partial resistance of BALB/c J provides a model system for studying the genetics of plasmacytoma susceptibility. A survey of genetic differences including over 50 genes (Rodericks' Alpha gene survey) only 1 difference (Qa-2 and Qa-3). It is also known that BALB/c An and BALB/c J have differences in genes controlling catecholamine biosynthesis. We are attempting to find new genetic differences by using DNA probes, and to this end have begun with retroviral gene probes. Differences have been found in flanking sequences around MTV and a xenotropic type c probe. These are being pursued as possible clues. We hope to make probes specific for the flanking sequences and then to use these as genetic markers.

A working hypothesis is that BALB/c is susceptible to developing plasmacytomas because (differentiation specific) genes controlling plasma cell proliferation are regulated in a special way. This could be caused by genetic events that involve the regulatory elements of these putative genes. Candidates for such events are retroviral gene insertion, or consequences of retroviral gene recombination with host genes.

Oil granuloma-growth promotion. The peritoneal oil granuloma provides an essential microenvironment for the growth of primary (early) plasmacytomas. This phenomenon resembles the growth dependency of certain endocrine tumors, e.g., estrogen dependent interstitial cell tumors of the testis. Attempts are being made to identify the factors involved in growth dependency.

In studies with Mr. R. Nordan primary plasmacytoma cells (PCT-C) have been propagated in millipore diffusion chambers (MDC). It has been possible to make preparations of primary plasmacytomas that contain a very few macrophages, and these have grown in MDC in normal and pristane conditioned mice. There has been a consistent increase in the proliferation of granuloma dependent primary PCT in MDC implanted in pristane conditioned mice as compared with normals strongly suggesting that diffusable substances influence primary plasmacytoma growth. Alternatively it is possible that normal peritoneal exudate cells produce inhibitors for primary plasmacytoma growth and that the injection of pristane in some way inhibits these cells. This possibility has not been ruled out. Further studies will attempt to better characterize these effects with the hope of determining the biochemical nature of the growth stimulating substance. Mr. Nordan has further shown that when primary plasmacytomas and certain long term transplantable plasmacytomas are put in millipore diffusion chambers and implanted into pristane conditioned mice treated with indomethacin growth is severely inhibited.

Immunochemistry and genetics of immunoglobulins

1. Vk-isotypes. The Vk structures produced in the BALB/c mouse have a diversity of structures in the first framework region of the chain (positions 1 to 23). Amino acid sequence analysis of this segment can potentially provide data on the number of Vk-genes in the BALB/c genome. Seventeen new Vk-partial sequences have been completed by S. Rudikoff. We have sent these sequences to Drs. E. Haber and John Newell, Massachusetts General Hospital, who are analyzing them for relatedness and the construction of a dendrogram based on the number of nucleotide differences. It is anticipated this dendrogram will provide a basis for predicting the total number of BALB/c-k genes.

2. Structure of galactan binding myeloma proteins (GALBMP). Complete V-region structures of four galactan binding myeloma proteins have been determined by S. Rudikoff. Some progress has been made by Dr. D.R. Davies and his group in solving the structure of one of these J539. Hypothetical model building offers new approaches to determining 3 dimensional structures. Models are made by modifying segments of V-regions utilizing when possible known secondary structures. A new approach is being attempted in studies with Richard Feldmann using a computerized program, in which 3 dimensional space filling models can be graphically depicted from α-carbon coordinates To derive a new Ig-V structure from a determined structure requires an analysis of the amino acid side chain contacts, in the substituted molecule Richard Feldmann has worked out a computer program for this process and has analyzed J539, and found a minimal number of contacts with all substitutions in place. The first J539 model has been completed. In collaboration with C.P.J. Glaudemans, two forms of the hapten β1,6 galactohexaose has been constructed from existing crystallographic data and we have fitted this hapten in the J539 binding site region. Currently we submitted this model for publication but are going on to make further refinements.

Models of the other GALBMP X24, X44, and T601, have been constructed and using a monoclonal antiidiotypic antibody to X24 (HyX24-14) prepared by Dr. M. Pawlita and Ms. E.B. Mushinski, we are currently mapping the idiotope. This monoclonal antibody has unusual properties. It not only recognizes an epitopic site that is unique to X24 but in addition a second cross-reactive site that is distributed on several other GALBMP. These two sites are about 15 Å apart and provide the basis for defining the binding site on HyX24-14. We plan this year to begin sequencing HyX24-14 with Dr. S. Rudikoff and to construct a model of it.

In collaborative studies with Dr. S. Smith-Gill who monoclonal antibodies have been prepared to hen egg white lysozyme (HEL). Using a battery of sequenced avian lysozymes from various species, it has been possible to localize the region on HEL contacted by the monoclonal antibody HyHEL-5, using 3-dimensional space filled models. We plan to extend this work by sequencing the V-regions of HyHEL-5 in collaboration with Dr. S. Rudikoff.

These two systems, the anti X24 idiotype and the anti-lysozyme system should provide models of antibody-protein interactions.

We continue the collaboration with Dr. M. Weigert on the genetics of the VK21 and VK19 groups. Using DNA probes to these Vk regions a number of genetic differences have been discovered in inbred and wild mice by Southern blot hybridization. Ms. C. Scott has prepared a probe for mouse λ-regions and is studying differences in λ-genes in wild mice.

Mammary tumorigenesis in wild mice

In collaborative studies with Dr. R. Callahan, NCI, wild mice in our colony were studied for mammary tumor virus genes. Two stocks were found to lack MTV genes, M. musculus musculus from Czechoslovakia and M. musculus breviostris from Morocco. These breeding colonies provide a new system for studying mechanisms of mammary tumorigenesis, by various types of carcinogens. Further, the role of specific types of mammary tumor viruses can be examined. In addition to these two stocks, Dr. Callahan has shown that M. musculus domesticus (Centreville Lights) from the Eastern Shore of Maryland, carry only one copy of an MTV genome that includes one of the LTR regions. These mice can be used to study the promoter-insertion theory in mammary tumorigenesis. Accordingly, the Centreville Lights are being expanded and mammary tumor incidence will be determined in virgin and breeding females. Further, another group will be injected with diethylstilbesterol pellets, or chemical carcinogens.

Through various crosses with inbred mice the chromosomal localization of the LTR-fragment in Centreville Lights will be sought.

Publications:

Julius, M.A., McKean, D.J., Potter, M., and Weigert, M.: Expression of kappa chains of the VK21 group in Mus musculus and related species. Mol. Immunol. 18: 11-18, 1981.

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Weiner, F., Babonits, M., Spira, J., Klein, G., and Potter, M.: Cytogenetic studies on IgA/lambda-producing murine plasmacytomas: regular occurrence of a T(12;15) translocation. Somatic Cell Genet. 6: 731-738, 1980.

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Finkelman, F.D., Kessler, S.W., Mushinski, J.F., and Potter, M.: IgD secreting murine plasmacytomas: Identification and partial characterization of two IgD myeloma proteins. J. Immunol. 126: 680-687, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08726-04 LCBGY

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Biological and Biochemical Characterization of Transplantation Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

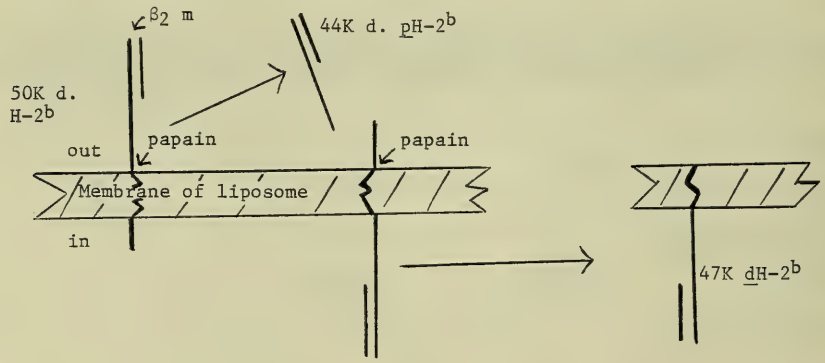
SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to examine the chemical and biological properties of murine histocompatibility antigens (H-2) and tumor associated transplantation antigens (TATA). The approach is to purify the molecules expressing these antigens and determine their properties at each stage of purification and when they are reconstituted into artificial membranes composed of lipid and other highly purified proteins. The properties examined include: 1) ability to induce in vivo transplantation rejection and humoral response, 2) ability to stimulate cytotoxic T lymphocytes in vitro, 3) chemical properties with particular emphasis on how these molecules bind to biological membranes, 4) the metabolism of these antigens in normal and neoplastic cells. The rationale for this approach is that by examining the behavior of these molecules in simple reconstituted membranes, an understanding can be gained of how these molecules interact with receptors on lymphoid cells in order to express their known biological effects on T cell mediated immune reactions (H-2). Furthermore, knowledge of the biological properties of these molecules will provide a rational basis for using them in in vivo therapeutic protocols.

Results :

I. Purification and properties of H-2^b

The purification of H-2^b and a preliminary characterization of artificial liposomes was already reported. In the earlier studies it appeared that some H-2^b was completely resistant to papain digestion. We have now determined that this resistance was due to some H-2^b being bound to multilamellar liposomes and thus buried in lipid and protected from proteolysis. When liposomes are prepared under conditions where mainly unilamellar liposomes are produced, only two forms of H-2^b were observed, both susceptible to papain digestion. About half of the H-2^b is oriented toward the outside of the liposome and is cleaved by papain to produce a 44,000 dalton fragment which is released from the liposome and contains all of the alloantigenic sites. The other half is oriented toward the inside of the liposomes and is cleaved by papain to produce a 47,000 dalton fragment that remains bound to the liposome. No intact 50,000 dalton H-2^b remains. The drawing below illustrates how H-2^b is bound to liposomes and how the various molecules are produced by papain.



Liposomes containing H-2^b have now been successfully used to stimulate primed, allostimulated spleen cells to produce cytotoxic T cells. Spleen cells from B10A (H-2^a) animals primed with i.p. injections of B10 (H-2^b) spleen cells were incubated for 5 days with liposomes containing H-2^b. After 5 days cytotoxic T cells were isolated from these incubations which could kill RBL-5 (H-2^b) tumor cells but were unable to kill YAC (H-2^a) tumor cells. The effects of 1) varying the density of H-2^b on the liposomes 2) varying the concentration of liposomes and 3) removal of adherent cells before secondary stimulation are under investigation by the Immunotoxicology Branch of Litton Bionetics.

In collaboration with Dr. Linda Gooding we are planning to expand these artificial liposome experiments to include another immunogenic protein incorporated into liposomes along with H-2^b. Human erythrocyte glycophorin has been chosen since it is immunogenic, has been shown to bind to artificial membranes and can be easily purified. Our lab at NIH will concentrate on defining the effects of glycophorin on the immunogenicity of H-2^b in the in vitro secondary stimulation experiments described above. Dr. Gooding will concentrate on the immunogenicity of glycophorin on these liposomes. Initially, liposomes containing glycophorin will be fused with spleen cells and these will be used as targets to search for cytotoxic glycophorin specific T cells in the spleens of animals immunized with either H-2 + glycophorin liposomes or glycophorin containing spleen cells.

II. Purification and properties of TATA

Two separate projects are in progress. A. In the first, partially purified TATA preparations from the highly immunogenic chemically induced tumors Meth A and CI-4 have been used in reconstitution experiments. Previous reports that the bulk of the Meth A TATA was localized in the cytoplasm were confirmed and extended. It was found that the TATA of CI-4 was also localized primarily in the cytoplasm. The soluble and membrane bound TATA activity from both CI-4 and Meth A were bound to artificial membranes and it was found that the immunogenicity of soluble TATA could be significantly enhanced by association with egg lecithin liposomes. This project is being continued in two directions. First, liposomes with bound TATA are being tested in immunotherapy protocols, i.e., the CI-4 and Meth A tumors are being administered to mice prior to injection of the liposomes. In these studies highly purified preparations of TATA now available by a technique developed by Dr. G. DuBois in E. Appella's laboratory will also be used in the liposomes. Second, the tissue culture line derived from Meth A has been cloned, and 20 individual clones are being tested for immunogenicity in the in vivo assay. It is hoped that variants will be found with the immunosuppressive activity that has been described for other methylcholanthrene induced tumors. These studies are ultimately aimed at discovering the reasons for the very strong immunogenicity of Meth A compared to other chemically induced tumors.

B. The other major ongoing project involving TATA is the study of alien H-2 antigenic specificities appearing on a chemically induced tumor. We are focusing on two tumors. DMLM 1678, a dimethylbenzanthracene induced lymphoma of H-2^s origin has been shown serologically to express a previously undiscovered public specificity cross-reacting with anti H-$2K^b$ antiserum. We are completing these studies by isolating the molecules precipitated by anti H-2^s and anti H-$2K^b$ and showing that they are biochemically identical.

Br-1, a methylcholanthrene induced tumor of H-2^k origin appears to express an alien H-$2D^b$ specificity. Although not a new public specificity since it is absent from normal Br-1 tissues, this antigen appears to be expressed on the normal H-2^k molecules of the Br-1 tumor. The already published preliminary biochemical characterization of this antigen is now being extended. Moreover, the Br-1 tumor is also being adapted to tissue culture and cloned with the idea of determining if this alien antigen is expressed on all of the Br-1 tumor cells or only a small subpopulation. This study is aimed at determining why this alien antigen does not appear to function as a TATA on this tumor.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08727-04 LCBGY

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Organization and Control of material in immunoglobulin-secreting plasmacytomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range purpose of this project to study the control mechanisms important in regulating protein synthesis in normal and malignant lymphoid cells. The organization of immunoglobulin light and heavy chain genes and the control mechanisms in selecting those being transcribed and those which are expressed in protein synthesis are being studied. Particular attention is being given to the molecular genetics of IgD, since this molecule, along with IgM, appears to be a very important surface component of most B lymphocytes. We have prepared a cDNA clone for mouse IgD heavy chain and used it to isolate a genomic DNA clone containing both IgD and IgM heavy chain genes. The DNA sequence of both clones has been completed, and we have discovered that IgD has multiple gene segments coding for alternative carboxyl-termini for IgD that either is secreted or membrane bound. The mRNAs for these different forms of IgD heavy chain have been identified and their processing is being studied.

PHS-6040
(Rev. 2-81)

Methods Employed:

These studies were done using the following tissues: BALB/c liver and spleens; BALB/c plasmacytomas and lymphomas; human chronic lymphocytic leukemia cells and cultured lymphoma and plasmacytoma cells. RNA was isolated using guanidinium thiocyanate or LiCl-urea, and DNA was isolated using SDS and proteinase K. Double stranded cDNA was prepared from RNA fractions enriched for particular messengers using reverse transcriptase, DNA polymerase I, and S1 nuclease. This cDNA was inserted into the Pst I site of pBR322 by oligo dC-oligo dG tailing and annealing, and the recombinant DNAs were used to transform calcium shocked E. coli. Colony hybridization with ^{32}P labeled mRNA or nick translated DNA fragments identified likely candidates for desired clones. Genomic clones were identified in Charon 4A and Charon 28 libraries of genomic DNA fragments using cDNA plasmid clones. DNAs were mapped using restriction endonuclease digestions and DNA sequence determination was performed using the Maxam-Gilbert techniques. mRNAs from cytoplasms and nuclei were studied by electrophoresis on methyl mercury hydroxide agarose gels, transfered to diazotized paper and hybridization to ^{32}P labeled probes.

Major Findings:

Structural studies of cDNA and genomic clones of BALB/c IgD heavy chain (δ chain) constant region demonstrated that the δ gene consists of exons which encode two constant region domains, Cδ1 and Cδ3, a hinge region CδH, separating the two constant domains, and at least 4 exons which appear to encode alternate 3' termini for different δ chain proteins such as those which are membrane bound and those which are secreted. In plasmacytomas TEPC1017 and TEPC1033, which secrete IgD as well as bear IgD on the cell surface, we have identified 4 RNAs that hybridize to the δ chain cDNA clone. The smallest of these δRNAs, containing 1750 nucleotides (NT) is the most abundant RNA and is felt to be the mRNA for secreted δ chains. The next most abundant RNA, containing 2900 NT, is felt to be the mRNA for membrane bound δ chains, because this is the most abundant δRNA in normal spleen cells. Hybridization studies of these RNAs using probes from the different genomic exons, as diagrammed below, indicate that RNA splicing attaches the DC gene segment to the secretory δ message of 1750 NT and the VVDC gene segment to the 2900 NT RNA for membrane bound δ chains. The minor bands consisting of 2100 NT and 3200 NT, contain VVDC and DC coding regions, respectively. The 3200 NT δRNA is probably the precursor of the mature 1750 NT mRNA, but the 2100 NT species remains a mystery. It could be a scarce, short-lived mature membrane δ mRNA derived from a 2900 NT precursor, or since it has not been detected in normal spleen RNAs it may be an RNA found only in tumors.

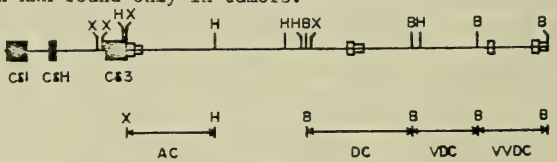


Figure 1. Restriction map showing the BALB/c δ gene and the genomic fragments subcloned from it. B = BamHl, H = Hind III, and X = Xba I.

TABLE 1. Sizes of δRNAs that hybridize with portions of the δ gene

	AC	DC	VDC	VVDC
TEPC 1017	3200 NT	3200 NT	----	2900 NT
	2900 NT	1750 NT	----	2100 NT
TEPC 1033	3200 NT	3200 NT	----	2900 NT
	2900 NT	1750 NT	----	2100 NT
BALB/c spleen	----	----	----	3200 NT
	----	----	----	2900 NT

Southern blot experiments with genomic DNAs indicate that in normal spleen cells and in μ + δ containing lymphomas the μ genes and δ genes retain their germ line configuration separated by 4600 bp of DNA. However, in TEPC 1017 and TEPC 1033 a VDJ region complex appears to have switched to a position between these genes causing deletion of the μ genes. This rearrangement is thought to take place in those rare instances when B cells differentiate to become IgD secreting cells.

Significance and Proposed Course

IgM and IgD constant region genes are located far closer to one another than those of any other Ig heavy chains. It appears likely that this entire μ + δ region may normally be transcribed into one very large RNA which is a precursor from which secreted μ , membrane μ , secreted, δ or membrane δ mRNAs can be produced by different RNA splices. This process will be studied in normal spleen cells and in μ + δ containing B-cell tumors. The RNA processing pathways in the IgD secreting plasmacytomas will be studied further to elucidate how the different δ mRNAs are created.

BALB/c δ chains seem to lack the second constant region domain that appears to be present in human δ chains. We plan to study the human δ gene by making cDNA clones from a human plasmacytoma in tissue culture and from leukemia cells which bear surface IgD. It will be important to see whether this Cδ2 is present in human DNA and other mouse genomic DNAs. In addition, the IgD leukemia cells we have collected are from a family in which 4 out of 5 siblings contracted chronic lymphocytic leukemia, generally expressing surface IgD. We hope to see if the genomic DNA of these patients shows any characteristic defects or rearrangements in the DNA near the δ chain gene.

We are also beginning a major study of the V-region genes for heavy and light chains of galactan binding antibodies. To this end we have constructed cDNA clones for kappa and alpha chain mRNAs from XRPC 24, and a shotgun library from XRPC24 tumor DNA has been constructed. The number of genes coding for this antigen binding specificity in BALB/lc and other mice will be studied.

Publications:

Mushinski, J.F., Koziol, J.A., and Marini, M.S.: Cluster analysis of aminoacyl-tRNAs from mouse plasmacytomas correlates chromatographic profiles with myeloma protein similarity, clonal origin of tumor lines, and the neoplastic nature of the tissues. J. Theoret. Biol. 85: 507-521, 1980.

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Tucker, P.W., Liu, C-P, Mushinski, J.F., and Blattner, F.R.: Mouse immunoglobulin D: Messenger RNA and genomic sequences. Science 209: 1353-1360, 1980.

Mushinski, J.F., Blattner, F.R., Owens, J.D., Finkelman, F.D., Kessler, S.W., Fitzmaurice, L., Potter, M., and Tucker, F.W.: Mouse immunoglobulin D: Construction and characterization of a cloned δ chain cDNA. Proc. Natl. Acad. Sci. USA 77: 7405-7409, 1980.

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ANNUAL REPORT OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1980 through September 30 1981

The clinical research program of the Metabolism Branch is directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. In this area, a broad range of immunological investigations are carried out in patients with malignancy, in patients with immune deficiency diseases and a high incidence of neoplasia as well as in experimental animal systems to determine the nature of immune processes that are of importance in the surveillance against neoplastic cells and to define the nature of defects in the immunological system that result in an increased incidence of neoplasia. Major efforts in this area are also directed toward: 1) Defining events of cellular maturation, cellular interaction and cellular biosynthesis involved in the normal circulating antibody response. Specific emphasis is placed on studies of the immunoglobulin genes and their rearrangements and deletions that control immunoglobulin synthesis and on the action of immunoregulatory cells including helper T cells, suppressor T cells and macrophages that regulate antibody responses and on studies of disorders of these immunoregulatory cell interactions in patients with immune dysfunction. 2) Identification of unique cell surface determinants on subpopulations of lymphoid cells with different functional capabilities using antibodies developed with hybridoma technology. 3) The genetic control of the immune response especially as related to the immune response genes associated with the major histocompatibility complex. 4) Studies of the mechanism of action of various cytotoxic cells and their contribution to host defense. 5) Detection of circulating tumor associated antigens. (6) The isolation and characterization of biological modifiers that suppress the human immune response that are produced by T cell lines and T-T cell hybridomas. The second major goal of the Branch is to determine the physiological and biochemical effects that a tumor produces on the metabolism of the host. Both patients with neoplastic disease as well as those with non-neoplastic disorders that facilitate the development of techniques for the study of cell membranes, homeostatic mechanisms and metabolic derrangements of biochemical control mechanisms are being investigated. Within this area a special emphasis is laid on the biochemical events accompanying normal growth and the hormonal control of this growth as it relates to our understanding of malignant growth and on the metabolism of proline and the porphyrins.

Immunoregulatory Cell Interactions in Immune Response

A major effort of the Metabolism Branch over the past few years has been directed toward defining the major events of cellular differentiation, cellular interaction and cellular biosynthesis involved in the specific circulating immune response. These studies have placed special emphasis

on defining the defects of immunoglobulin genes including their patterns of rearrangement and deletion that regulate B cell differentiation that occur in patients with primary immunodeficiency diseases associated with a high incidence of malignancy, in patients with autoimmune disorders as well as in patients with malignancies of the T or B lymphocyte systems. Overall these studies were directed at defining the factors in normal and abnormal states controlling the production of antibodies and the synthesis of the immunoglobulin molecules. Cells that ultimately produce antibodies undergo sequential maturation from stem cells in the marrow to B lymphocytes and then from B lymphocytes into immunoglobulin synthesizing plasma cells.

The earliest events of maturation of stem cells into B cells involve rearrangements of the genes coding for the appropriate light and heavy immunogloublin chains. These events have been examined for human cells in collaboration with Dr. Philip Heiter and Dr. Phillip Leder. Human light chain genes exist as discontinuous gene segments of V (variable), J (joining), and C (constant) regions in the germline state. During maturation of stem cells into B cells a rearrangement occurs in the expressed gene bringing a single V region in contiguity with a specific J region. ^{32}P labeled clones of human kappa (C_K) and lambda ($C\lambda$) constant region genes were used as probes to study gene rearrangements in lymphocytes from chronic lymphocytic leukemia patients, Epstein-Barr virus transformed and spontaneous B-cell lines. Genomic DNA was extracted from these cells digested with the restriction endonucleases known to demonstrate both rearranged and germline alleles, and hybridized with the probes. C_K exists as a single gene in man. The BamHI digests of all 8 kappa expressing B-cells demonstrated at least one rearranged kappa allele. Four displayed a remaining germline allele, while 2 had double rearrangements and 2 had deleted the other allele. There are thus three different patterns accounting for allelic exclusion. The excluded allel may be germ line, deleted or rearranged presumably in an aberent fashion. At least six $C\lambda$ genes were shown to exist in each individual in at least two germline, patterns due to polymorphism of EcoRl restriction sites. EcoRl digests of all 11 lambda expressing B-cells showed a rearranged lambda allele (3 displayed double rearrangements) not seen in fibroblasts or T-cells from the same individual. When genes coding for the opposite isotype were examined Kappa expressing B cells retained their lambda genes in the germ line configuration. However, lambda expressing B cell deleted or rearranged all of their kappa consistant region genes. Such kappa gene deletions usually involved J_K as well as C_K region but spared a V_K family. These observations suggested an ordered hierarchy with kappa gene activation preceeding lambda. In contrast to B cells, human T cell leukemias and lines almost always retained their immunoglobulin genes in the germ line configuration. In order to study the earliest events of immunoglobulin gene recombination we have examined eight human "non-T, non "B" leukemic lymphocytes representing immature stages of lymphoid differentiation. Fully seven of these eight populations demonstrated immunoglobulin gene rearrangements indicating that most cases of this leukemia are already committed to B-cell development at the immunoglobulin gene level. In addition, the majority of heavy and light chain gene rearrangements observed were not accompanied by detectable cytoplasmic immunoglobulin suggesting that many of these

rearrangements may be of an aberrant nature. Such populations of B-cell precursors, rich in recombinational errors of immunoglobulin gene joining, may indicate that the somatic assembly of immunoglobulin genes is remarkably prone to error. Furthermore, the patterns of immunoglobulin gene rearrangement within these leukemic pre B cells suggests a hierarchy of somatic rearrangements with mu genes preceeding light chains and kappa light chain genes generally preceeding lambda.

The maturation of B cells into antibody producing plasma cells is carefully regulated both positively and negatively by distinct subpopulations of lymphoid cells. Specifically many antigens and mitogens require the presence of both helper lymphocytes of thymic origin (helper T cells) and macrophages as well as the B cells to induce a full antibody response. More recently it has been recognized that a separate class of thymic derived cells, suppressor T cells, may act as negative regulators of B cell maturation inhibiting this process. We and others have shown that suppressor T cells emerge from the thymus as inactive prosuppressor cells that require an interaction with another T cell (termed a suppressor inducer or activator) in order to become a final effector of suppression. To study these events we have developed a series of techniques including techniques to study the terminal differentiation of B cells into immunoglobulin synthesizing and secreting cells, techniques to assess helper T cell function, and to detect both increased and decreased functional activities of suppressor effector T cells and their precursors and activators. Previously we used a single basic procedure for these determinations in which peripheral blood lymphocytes were cultured *in vitro* with pokeweed mitogen, a polyclonal activator, and the IgG, IgA, and IgM synthesized and secreted by these cells was quantitated using radioimmunoassay procedures. Pokeweed mitogen is a prototype of a thymic and macrophage dependent activtor of B cells that also has the capacity to activate some prosuppressor cells to effectors of suppression.

Much has been learned in recent years in this and other laboratories about the regulation of the immune response by studying total immunoglobulin production following stimulation *in vitro* by polyclonal activators. To extend this work and to more closely approximate the *in vivo* situation, we have initiated studies of immunoglobulin class specific antigen-specific antibody production that do not require polyclonal B cell activators. A major new accomplishment over the past year in this area has been the development of a culture and assay system for the sensitization of human peripheral blood mononuclear cells with a T cell dependent antigen, sheep erythrocytes, in the absence of nonspecific stimulatory agents and with the subsequent generation of macroscopic hemolytic plaques. We have shown that the antibody produced by the plaque forming cells generated in this culture system is specific for the sensitizing antigen, and that the plaques created are not false plaques since their formation is inhibited by cycloheximide. In addition we have shown that the antigen specific response measured by this system is dependent on adherent cells and T lymphocytes. At least one population of the helper T cells is sensitive to 2000R irradiation. This system is simple, sensitive, and should serve as an effective tool for the analysis of cellular interactions involved in the generation of human antigen specific PFC, the genetic control of the human immune response, and the pathophysiology of altered immunoregulation in disease.

We have also developed and utilized an alternative method to measure specific antibody responses by human peripheral blood mononuclear cells in vitro and have used this method to study defects in the immune responsiveness of patients with various immunodeficiency states. For these studies, we have employed influenza viruses as antigens. Antibody to influenza virus produced by cells in culture is measured by an Enzyme-Linked ImmunoSorbent Assay (ELISA) which utilizes the substrate to produce conversion by alkaline-phosphatase conjugated antibodies to human immunoglobulins as an amplifying probe. Cultures of peripheral blood mononuclear cells from >95% of normal adult individuals produce specific anti-influenza virus antibody when washed free of cytophilic antibody and cultured 10-12 days in vitro in the presence of type A influenza viruses. Antibody secretion requires de novo protein synthesis, begins about day 5 of culture and reaches maximal rates between days 5 and 7 of culture. Antibody synthesis can be induced by both live influenza type A and B viruses as infectious allantoic fluid or formalin inactivated, zonally purified type A and B viruses. Antibody generation was both antigen dependent and virus specific at the induction phase since: 1) cultures in media alone or stimulated with normal allantoic fluid produced no antibody and 2) cultures stimulated with type A viruses produced anti-influenza A antibody but not anti-influenza B antibody and vice versa. The production of antibody was shown to require the cooperative interaction of T-cells, B-cells and monocytes in culture. This requirement for T helper cells capable of promoting the maturation of B-cells into antibody secreting cells was further investigated using a series of hybridoma derived monoclonal antibodies directed at predominantly distinct subsets of human T-cells.

Using this methodology the human T-helper cell for anti-influenza antibody responses bore specificities recognized by the monoclonal antibody OKT4 but not specificities recognized by the monoclonal OKT8 which recognizes virus immune CTL effectors. Thus a subset of human T-cells comprising 40-60% of the total T-cell population was identified as the helper cell subset for specific antibody responses by B-cells in vitro.

Studies have been initiated to utilize this method to study maturational and immunoreguatory abnormalities in patients with immunodeficiency diseases. Three patients studied with X-linked hypogammaglobulinemia and isolated growth hormone deficiency failed to produce antibody in vitro in spite of having the capacity to mount a specific cytotoxic T-cell response to the same virus. In addition all 3 patients' cells could provide help to normal allogeneic B-cells and did not inhibit antibody production by cells from normal individuals. Thus these patients have an immune defect involving antibody secreting cells or their precursors. Of eleven hypogammaglobulinemic patients studied with a different disease termed common variable hypogammaglobulinemia (CVH), five made measurable antibody responses in vitro. Since CVH patients make specific antibody responses poorly in vivo, the finding of intact in vitro antibody responsiveness suggests that some of these patients have a host environmental abnormality as the cause of their hypogammaglobulinemia which can be overcome in vitro. Specific antibody responses were also sought in patients with ataxia-telangiectasia and the Wiscott-Aldrich syndrome who lacked influenza specific CTL responses. Six of seven patients with the Wiskott-Aldrich syndrome failed to produce specific antibody as did 4 of 5 patients with ataxia-telangiectasia.

We have extended our investigation of Epstein-Barr virus as an activator of human B cells into immunoglobulin secreting cells. The Epstein Barr virus (EBV) is the etiologic agent of infectious mononucleosis and has been implicated in the pathogenesis of African Burkitt's lymphoma and nasopharyngeal carcinoma. EBV, like most viruses of the herpes group, becomes integrated into the genome of the host cells, and persists there for life. EBV is of particular interest to immunologists since it infects cells of the immune system almost exclusively, specifically the B lymphocytes. We have found that B lymphocytes infected with EBV become activated to proliferate and differentiate to actively secrete immunoglobulin and antibodies. After primary infection, EBV immune T cells appear which are capable of inhibiting or suppressing further B cell activation by EBV. Several recent lines of evidence suggest a potential relationship between EBV and the disease rheumatoid arthritis (RA). Patients with RA have elevated serum antibody titers to certain EBV associated antigens, EBV rapidly induces B cell lines from the peripheral lymphocytes of RA patients and EBV infected B cells produce rheumatoid factor in culture. We have studied immunoregulatory T cell function in 20 patients with RA to detemine whether a defect in the function of such cells might be present in this disease. Helper T cell function for pokeweed mitogen induced immunoglobulin production was normal in these patients as was the suppressor T cell function of RA cells activated by allogeneic histocompatibility antigens. Strikingly, however, the EBV specific suppressor T cell which inhibits EBV induced B cell activation in normal immune subjects was markedly deficient in these RA patients even though they were immune to EBV as determined by the presence of antibodies to EBV in their serum. Thus, patients with this disease exhibit a profound defect in immunoregulatory T cell function which is restricted to involve the response to only a specific agent the EBV. Since EBV persists indefinitely in the genome of B lymphocytes after primary infection and since this latent virus can activate B cells to produce immunoglobulins, it represents a potential perpetual stimulus for production of antibody and rheumatoid factor. Thus, the defect in the specific immunoregulatory T cells which prevent EBV induced B cell activation may be an important contributor to the immunologic basis of this common disease.

The mechanisms and sites of action of suppressor T cells have been a major area of interest in many laboratories. In murine systems, many suppressor T cells which inhibit B cell function appear to do so by neutralizing or inactivating helper T cells required for the particular B cell response being measured. In most of the suppressor cell assays useful for studies of human B cell responses, the site of action of the suppressor cell is unknown. The Epstein-Barr virus offers a unique tool to address this issue since it is capable of activating B cells in the complete absence of helper T cell influences. Thus, if suppression of EBV induced immunoglobulin production is observed, such suppression must be acting directly on the B cell. We have been able to define four separate and distinct suppressor T cell systems, and all four of these suppressor T cells act directly on the B cell since they suppress the EBV induced response of these cells. In infectious mononucleosis, suppressor T cells are present which inhibit all B cell activators, both helper T cell dependent ones such as PWM, and helper cell independent activators such as EBV. In the human neonate, such polyclonal suppressor cells are not found circulating in an activated state, but after stimulation with PWM they become activated and also then inhibit EBV induced responses.

In EBV immune subjects, we observed a unique late acting suppression which was specific for EBV alone, not inhibiting PWM induced responses. This suppressor T cell then also acts only on B cells. The fourth suppressor system was that observed in cultures containing mixtures of cells from individuals who differed significantly in their major histocompatibility antigens. In this circumstance suppressor T cells become activated which inhibit the EBV, but not the PWM, induced B cell response. Again, this suppressor mechanism must act directly on the B cell, but in this case, only that subpopulation of B cells which responds to EBV.

Using the in vitro biosynthesis procedures with polyclonal activation we developed we have identified patients with disordered immunoglobulin synthesis due to a variety of mechanisms including disorders of intrinsic B cell activity, disorders of helper T cell function, disorders of the network of interacting T cells involved in immune suppression as well as disorders of monocyte function. Patients with hypogammaglobulinemia with primary B cell defects that we have defined include patients with x-linked agammaglobulinemia, patients with certain types of common variable immunodeficiency, with selective IgA deficiency or with the immunodeficiency characterized by elevated IgM levels and reduced IgG and IgA levels.

A second area of interest has been in helper T cell activity in animal models and in patients with disorders of immunity. In clinical studies deficiency of helper T cells was observed in patients with severe combined immunodeficiency disease with B cells and a patient with common variable hypogammaglobulinemia and in some patients with ataxia-telangiectasia. An extreme defect in helper T cells was shown to be the cause of the hypogammaglobulinemia in the form of severe combined immunodeficiency characterized by an absence of circulating T cells but normal numbers of B cells. The B cells of these patients could not synthesize immunoglobulin molecules when cultured with pokeweed mitogen in vitro but synthesized immunoglobulin normally when normal T cells were added to the culture system. The primary defect in such patients might be a defect of T stem cells or to a failure of thymus function leading to a failure in the maturation of such stem cells into functional T cells. A similar pattern of hypogammaglobulinemia with normal B cell function has been identified in a patient with common variable hypogammaglobulinemia who has a T cell defect limited to the helper T cell series.

The technique for assessing helper T cell activity were applied to homogenous populations of T cells from patients with T cell leukemias. One of 20 acute T cell leukemias and eight of 16 patients with Sezary T cell leukemias retained the capacity to help normal B cells in pokeweed mitogen stimulated cultures. It is of interest that these patients had high IgA and IgE levels and in 11 cases the Sezary cell leukemia has been associated with a circulating monoclonal immunoglobulin.

We are preparing monoclonal antisera to the idiotype of the heavy chains of these monoclonal immunoglobulins to determine if they react with the putative T cell receptors since it has been suggested that helper T cells for specific B cell clones share immunological determinants with the hypervariable region of the heavy chain of the specific antibody whose production is stimulated by the T cells.

Over the past year we have analyzed leukemic T cells with retained functions with monoclonal hybridoma antisera that define T cell subsets. Some of these antisera obtained commercially define T cell subsets with different functions (e.g. OKT 4, helper T cells, OKT 5, 8 suppressor/cytotoxic T cells) whereas others define the state of T cell maturation (e.g. OKT 10 immature cells, OKT 3, mature T cells, anti-Ia, activated T cells). We have also used a monoclonal hybridoma anti-T cell antibody we have prepared ourselves that defines a 120,000 mol. wt. antigen termed Tac on the surface of activated T cells. Normal thymocytes and circulating T cells do not bear this antigen whereas T cells activated with mitogen, the mixed leukocyte reaction, or T cell growth factor do. All acute T cell leukemias bear the OKT 10 antigen but rarely bear the OKT 4 or 5 antigen, and in only 5% of cases the OK 3 maturation antigen. The seven Sezary leukemia cells studied in contrast do not bear OKT 10 but are all OK 3 and OKT 4 (e.g. mature helper phenotype) positive and are OK 5, 8 (e.g. suppressor phenotype) negative. They are also negative for Ia and Tac antigens. Thus the studies with monoclonal antibodies is in accord with our previous conclusions that Sezary cells are relatively mature T cells that are dedicated to helper interactions with B cells.

We have demonstrated excessive suppressor T cell activity in the neonate and in association with a variety of immunodeficiency states including infectious mononucleosis, one form of T cell leukemia, a subset of patients with common variable hypogammaglobulinemia, most patients with thymoma and hypogammaglobulinemia and some patients with selective IgA deficiency.

Studies on three patients with T cell leukemias with retained suppressor activity have been continued in order to address one of the most critical issues concerning the capacity of T cells to regulate human immunoglobulin synthesis, that is whether suppressor cell activity is generated by a single population of T cells acting alone or whether suppressor cell generation requires interaction between different populations of T cells neither of which has the capacity to affect suppression alone. The proof of such T-T interaction requires clear identification and separation of the two classes of cells involved. In human systems, this has been exceedingly difficult until now. We have taken advantage of an unusual patient with acute lymphoblastic leukemia and hypogammaglobulinemia prior to therapy to address this issue. In our studies we learned that the neoplastic T cells from this patient functioned as pro-suppressor cells. These cells were OKT 10 positive but negative for the other antigens discussed above. We observed that the patient's leukemic T-cells produced no detectable immunoglobulin and depressed the immunoglobulin production of co-cultured normal unseparated lymphocytes by 85-100% in the presence of pokeweed mitogen. However, suppression was observed only when cooperating normal T cells and pokeweed mitogen were present. Prior irradiation of either the leukemic cells or the cooperating normal cells nulified the suppressor effect. We, thus, were able to conclude that an interaction between at least two different T cell subsets is required for the generation of so-called suppressor-effector T cells in man. We extended these studies by analyzing whether normal T cells could secrete a soluble factor which could activate the leukemic cells under discussion to become suppressor effector cells. We learned that normal T cells when stimulated with

pokeweed mitogen secrete a factor (or a series of factors) which may activate the leukemic cells to become direct suppressors without the further need of cooperating T cells in a pokeweed mitogen driven system of immunoglobulin production. In a Nocardia water-soluble mitogen-driven system of immunoglobulin production (known to be relatively T-independent), it was learned that both pokeweed mitogen and these leukemic cells were required for suppressor effects. These studies enabled us to construct a model for the induction of human suppressor effector cells. In this model, pro-suppressor T cells must interact with a different activating set of T cells before becoming fully functional suppressor T cells. To our knowledge, these studies represent the first proof of cooperative interaction between neoplastic and normal T cells in the generation of immunoregulatory effector T cells. Furthermore, we showed that there was an alteration of the surface phenotype of the leukemic cells following incubation in vitro with the T cell inducing factors that paralleled the changes in function. Following incubation the leukemic cells became OK3 (mature phenotype) OK8 (suppressor phenotype) and Tac (activated phenotype) positive.

Another area of research has been directed toward isolating and characterizing soluble biological modifiers of the human immune response. Purified human peripheral blood mononuclear cells stimulated with the mitogenic lectin Concanavalin A (Con A), continuous cultures of human T cells grown in interleukin II as well as human T-T cell hybridomas have been prepared that elaborate a variety of immunoregulatory molecules including suppressor factors. We find evidence for at least two diffeent suppressor activities in the supernatants of these cells one of which negatively modulates in vitro immunoglobulin biosynthesis while the other inhibits T cell proliferation. Immunoglobulin production was studied using a pokeweed mitogen driven reverse hemolytic plaque assay. The humoral suppressor factor produced 40-80% inhibition of polyclonal antibody synthesis. This factor was: 1) of molecular weight 30-45,000 daltons, 2) noncytotoxic, 3) present as early as 8 hours after exposure to Con A, 4) reversed by the monosaccharide L-rhamnose but not by a variety of other simple sugars including alpha-methyl-D-mannoside, 5) produced by macrophage depleted T cell populations but not by B cells, 6) found in the supernatants of long term human T cell cultures. The second suppressor factor produced 40-85% inhibition of in vitro lymphocyte proliferation in response to the mitogenic lectins phytohemagglutinin and Con A and the antigens streptokinase-streptodornase and tetanus toxoid. This factor was also of molecular weight 30-45,000 daltons and noncytotoxic. Its effect, however, is blocked by N-acetyl-D-glucosamine but not by L-rhamnose. In contrast to the humoral factor, the production of this factor required the presence of macrophages. We conclude that activated suppressors elaborate different soluble factors which independently modulate humoral and cellular immune reactions.

A modification of the immunoglobulin biosynthesis procedure has been developed to assess the ability of macrophages to act as one of the helper cells required for B cell maturation and to detect activation of monocytes into cells that inhibit immunoglobulin synthesis. Optimal immunoglobulin synthesis induced by pokeweed mitogen, staphage lysate and streptolysin O were dependent on the

presence of macrophages. If excessive numbers of normal macrophages (i.e., over 50% of the cells in the culture system) profound suppression was observed. T cells were not involved in this suppression as participants or targets. The suppression does not act via cytotoxicity. Thus it appears that normal macrophages can be activated to inhibit B cell maturation and immunoglobulin synthesis. Patients with multiple myeloma have a disorder of the monocyte suppressor system with excessive numbers of activated suppressor monocytes that lead to the reduced polyclonal immunoglobulin synthesis observed with these patients. We have demonstrated a similar monocyte suppressor disorder associated with a monocyte produced suppressor factor in mice bearing transplantable myeloma tumors.

Another area of research during the past year has centered around the capacity of antibodies to Ia-like antigens to inhibit a variety of immune responses. In the mouse, Ia antigens are defined as the surface membrane determinants (usually glycoproteins) encoded by genes within the I-region of the H-2 locus. It is thought that Ia antigens are critically involved in a number of genetically determined immune responses and in a variety of T-cell regulatory control mechanisms. In man, Ia-like antigens exist, and they are encoded by genes associated with the HLA-D region. It is been known for several years that antibodies to Ia antigens can inhibit both cellular and humoral immune reactions. However, a precise understanding of all mechanisms for this effect has not been achieved.

We have developed evidence that at least one of the mechanisms involved in the inhibitory activity for both T cell proliferation and immunoglobulin synthesis of anti-Ia antibodies is the induction of suppressor T cells. This activation of suppressor T cells appears to depend initially upon an antibody-related modification of auxilliary macrophages, which secondarily brings about the activation of suppressor T cells. An antibody with an intact Fc region is necessary for this phenomenon. Since antibodies to Ia-like determinants may be detected in a variety of clinical circumstances (e.g., allotransplantation, cancer, maternal/fetal interactions, etc.) we believe these observations are of both clinical and theoretical interest. These findings also suggest potential therapeutic uses for such antisera as a tool for suppressing immune response.

Genetic Control of the Immune Response

Over the past 15 years the multilayered regulation of the immune system has become increasingly apparent, concurrently with a heightened recognition of the importance of disorders of the regulation of the immune system in many disease states. One of the most intriguing levels of such regulation is the genetic one, especially by genes within the major histocompatibility complex (MHC), encoding the major transplantation antigens. Most of these immune response or Ir genes in the mouse, studied primarily with simple synthetic antigens, have been found to be encoded in a region, designated the I region, within the murine MHC known as H-2. However their mechanism of action remains unknown despite intensive investigation.

We have studied the in vivo serum antibody response and in vitro T-lymphocyte proliferative response to a natural protein antigen, sperm whale myoglobin (Mb), and shown that both of these responses are controlled by the same Ir genes. Using inbred strains of mice which differ only in the H-2 complex (known as congenic strains), we demonstrated that two different genes, mapping in distinct subregions (I-A and I-C) of the I region of H-2, controlled the responses to different antigenic determinants (distinct chemical sites) on the same antigen molecule - the first example of such independent control of responses to different chemical moieties on the same protein molecule. Moreover, the gene which controlled the T-cell proliferative response to a given determinant of myoglobin also controlled the production of antibodies specific for that same region of the molecule - an indication of parallel control of T-lymphocyte recognition and activation of B lymphocytes with similar specificity. In the T lymphocyte proliferative response, the choice of which Ir genes functioned depended on the source of the macrophage which displayed or "presented" the antigen. Since the Ir genes were determinant-specific, the genotype of the macrophage also determined which antigenic determinants would stimulate T cells - explaining a process previously denoted "determinant selection." For instance, if (high responder x low responder)F_1 T cells immune to whole myoglobin were stimulated by fragments of myoglobin with antigen presenting macrophages from the high responder parent (with both Ir genes), the low responder parent (with neither Ir gene), or recombinants bearing only one or the other gene, the same F_1 T cells responded only to those fragments to which the mouse providing the macrophages could have responded.

The limitation of T cell proliferative responses is that they involve only T cells and macrophages, not B lymphocytes which produce antibody. In order to study the mechanisms of determinant-specific Ir gene control of the antibody response to myoglobin in vitro, we have now developed a modified Mishell-Dutton culture system in which secreted antibody specific for myoglobin can be measured in the culture supernatants by a solid phase radioimmunoassay. By this method, we can also measure the fine specificity of the supernatant antibodies for different fragments of myoglobin. The in vitro spleen cell antibody response to sperm whale myoglobin was found to be controlled by the same Ir genes which controlled the in vivo response, at the level of individual determinants on different fragments of myoglobin.. In addition, we have extended the mapping to recombinant H-2 haplotypes not previously studied. A non-H-2-linked effect, which had operated in vivo, also operated in vitro - an indication that it did not involve clearance of antigen or antibody in the whole organism. We have found this in vitro antibody response to be macrophage and helper T-cell dependent, as shown by experiments depleting these cells, and reconstituting with purified T cell or macrophage populations. In order to dissect the cell interactions involved in mediating this Ir gene control, we have mixed cell populations from different strains, in combinations which would not produce an allogeneic effect. Myoglobin-immune helper T cells from (high responder x low responder)F_1 hybrid mice would help T cell-depleted populations of B cells plus macrophages from myoglobin immune high responder mice but not myoglobin immune low responder mice. However, when macrophages alone were depleted from the F_1 spleen cell populations of T and B cells, macrophages of either the high responder parent or low responder parent could equally well reconstitute the response. Thus, while we cannot exclude the possibility that residual F_1 macrophages are presenting antigen, we have been unable to demonstrate an Ir genetic restriction on macrophage function

in this in vitro secondary antibody response. Therefore, since we did observe an Ir restriction for B cells plus macrophages, it is possible that in the in vitro secondary antibody response to myoglobin, Ir gene function is involved in T cell-B cell interactions but not in macrophage-lymphocyte interactions.

In order to identify the structural features of sperm whale myoglobin that make it immunogenic in high responders and nonimmunogenic in low responders, we studied the Ir gene control of the response to myoglobins. The antibody response to equine myoglobin, which shares the three-dimensional structure and 87% of the sequence of sperm whale myoglobin, was found to be under completely different Ir gene control from that to sperm whale myoglobin. Mice that were low responders to sperm whale myoglobin were high responders to equine myoglobin. Moreover, the Ir genes mapped in different loci. The antibody response to equine myoglobin was controlled by complementing Ir genes in I-A and H-2D. We were surprised to find an Ir gene for an antibody response to a soluble protein antigen mapping in H-2D, rather than the I region, since none had mapped there before. Thus, either the mechanism of action of this Ir gene is different from those in the I region, or equine myoglobin is the first soluble protein antigen recognized in association with a class I (H-2K/D) rather than Ia antigen.

To understand the molecular mechanism of this Ir gene control, one would like to understand the nature of the receptors for myoglobin on B cells and T cells. As a model of the former, we raised monoclonal hybridoma antibodies against sperm whale myoglobin. Although these had a high affinity (10^9 M^{-1}) for native myoglobin, all six monoclonal antibodies studied in detail failed to bind to any of the three CNBr cleavage fragments which together span the whole sequence of myoglobin. Therefore, they must have been highly specific for the native conformation. Since we could not use fragments to define the antigenic determinants recognized by these monoclonal antibodies, we used a series of 15 native myoglobins of similar three-dimensional structure and known amino acid sequence differences. By correlating the relative affinity, measured by competitive radioimmunoassay, with the amino acid substitutions, we were able to identify critical residues recognized by three of the monoclonal antibodies. Two of these recognized clusters of residues on the surface of myoglobin which were far apart in the primary sequence but brought together by the folding of the native molecule. These, termed topographic determinants, accounted for the failure to bind random conformation fragments of myoglobin. The residues identified were also largely outside the previously reported sequential determinants of myoglobin. The monoclonal antibodies were sensitive to subtle changes within antigenic determinants, such as the replacement of glutamic with aspartic acid.

Cellular Mechanisms Regulating Normal Mucosal Immune Responses and Disorders of Immune Regulation in Gastrointestinal Diseases

In recent years we have been concerned with the cellular mechanisms regulating mucosal immune responses in general and IgA immunoglobulin responses in particular. In prior studies we have shown the regulation of the IgA response was independent of the IgG and IgM responses. To determine the cellular basis of this observation we have begun investigating the properties of cloned regulatory T cells maintained in continuous

culture with interleukin 2 (IL-2) a T cell growth factor. In these studies we first established non-cloned Concanavalin A-stimulated T cells obtained from small intestinal Peyer's patches, colonic Peyer's patches and spleens of Balb/c mice. When such cells were able to survive in culture with periodic replacement of IL-2 (but without re-stimulation with Con A) we cloned each of them by limiting dilution and ultimately obtained two Peyer's patch T cell lines (obtained from separate cell sources), two spleen T cell lines (also from separate cell sources) and one colonic Peyer's patch T cell line. Clonal frequency analysis determined by limiting dilution and Poisson distribution indicated that each clone had in fact originated from a single cell. Analysis of re-cloned cells supported this conclusion.

The characteristics of these cloned lines was then tested with the following results: 1) the cloned T cells all bear Thy 1.2, LyT 1.2 and Ia ($I\text{-}A^d$ and $I\text{-}E^d$) as well as H-2 ($H\text{-}2^d$ K/0) surface antigens; the cells are non-specific esterase negative, non-phagocytic and surface and cytoplasmic Ig negative; finally, the cells are non-adherent to nylon-wool columns; 2) the cloned T cells can be stimulated to proliferate by Con A or PHA, but not LPS; 3) the cloned T cells have no capacity to lyse eight types of syngeneic, allogeneic and xenogeneic targets, i.e., are not cytotoxic effector cells; 4) the cloned cells cannot mediate ADCC and 5) the cloned T cells have an interesting distribution of Fc receptors: approximately 60% of the cells from each Peyer's patch derived clones bear IgA-Fc receptors whereas none of the spleen-derived clones or colonic Peyer's patch clone bears this receptor. Some of the cells in the Peyer's patch-derived clones also bear IgG-Fc receptors (40%) as well as IgM-Fc receptors (1-2%). The cells in the spleen and colonic Peyer's patch clone bear only IgG-Fc receptors when they bear any receptor at all.

Having characterized the cloned cells in this manner we proceeded to test their capability to serve as helper cells for inducing production of IgA and other immunoglobulins. We found that each of the cloned T cell, regardless of their origin and thus regardless of their IgA-Fc receptor status, was capable of helping B cells produce IgA. Thus IgA-Fc receptors are not necessary to help IgA synthesis. In preliminary studies we also tested the capacity of cloned T cells to initiate suppression of Ig synthesis and found that when such cells are mixed with fresh T cells they do induce suppressor cell activity. However, the class specificity of this suppressor cell activity is not yet known.

Crohn's disease is an inflammatory disease of the small bowel and/or large bowel characterized by ulceration and granuloma formation. In prior studies of Crohn's disease (CD) we have explored the possibility that this disease results from or is complicated by an abnormality in the regulation of the immune response. More specifically we have shown that many CD patients with relatively mild or inactive disease have in their peripheral blood a "covert" suppressor T cell which exerts profound suppressor cell activity detectable in vitro by the use of indicator cultures consisting of allogeneic purified B cells and irradiated T cells driven to produce Ig by the polyclonal stimulant pokeweed mitogen (PWM). Of great interest was the fact that the suppressor T cell was not manifest in unseparated cell populations, but only after purification

of T cells on Sephadex-anti-F(ab')$_2$ columns; hence the term "covert" suppressor T cells.

During this period we showed that the covert suppressor T cells could be active *in vivo* as well as *in vitro*. In these studies we investigated several patients who had both Crohn's disease as well as hypogammaglobulinemia. First, we showed that these patients' peripheral blood contained overt suppressor T cells which were manifest prior to cell separation on anti-F(ab')$_2$ columns as well as after cell separation. Second, we showed that these patients' B cells were capable of PWM-driven Ig synthesis providing allogeneic normal T cells were added to the cultures of the patient's B cells. More importantly, we showed that these patients' B cells were stimulated to produce Ig when a stimulant not requiring T cell help, EB virus was added to the culture. Thus, there was no defect in the patients' B cells and the hypogammaglobulinemia could indeed be attributed to an overt suppressor T cell.

These studies clearly demonstrate that immunoregulatory abnormalities are associated with patients with relatively mild Crohn's disease. Furthermore they show that the immunoregulatory abnormality can be manifest *in vivo* as well as *in vitro* and cause frank hypogammaglobulinemia.

Primary biliary cirrhosis (PBC) is a progressive inflammatory disease of the liver which leads to intrahepatic cholestasis, cirrhosis and, eventually, hepatic failure. It occurs mainly in females and is not susceptible to treatment with corticosteroids. In previous studies of this condition we found that when cell cultures containing syngeneic mixtures of purified B cells and T cells obtained from PBC patients were set up, the T cells failed to manifest the suppressor cell activity on PWM-stimulated Ig synthesis that is normally manifest at high T cell/B cell ratios.

Because we feel that autologous cell interactions are very important in the cellular abnormality in PBC we have now begun to investigate the cellular basis of the autologous mixed leukocyte reaction (AMLR). In these studies we first determined the functional capabilities of the cells activated in the AMLR. In these studies, purified T cells were exposed to B cells or macrophages to elicit an AMLR in a primary culture of autoreactive T cells. The T cells thus obtained were then re-isolated from the primary culture and added to a secondary culture composed of B cells alone plus PWM to measure helper effects of the autoreactive T cells or to a secondary culture composed of B cells and fresh irradiated T cells plus PWM to measure suppressor effects of the autoreactive T cells. In these systems B cells alone would not be expected to be induced to synthesize Ig by PWM without addition of helper T cells whereas B cells and irradiated T cells would produce optimal amounts of Ig and would, therefore, be susceptible to suppressor effects of activated T cells.

These studies point to the conclusion that the autologous MLR results, at least in part, from surface antigens expressed on cells (B cells) after cell activation. In addition, they show that the main functional consequence of the autologous MLR is the activation of a suppressor T

cell population. In the light of these findings it is reasonable to suggest that through the autologous MLR, cell activation leads to feedback suppressor induction and that in the autologous MLR one has a polyclonal equivalent to feedback suppressor circuits previously described in antigen specific systems.

These new observations help explain the defect in PBC. We now interpret the inability to generate a autologous MLR in this condition as an inability to generate feedback suppressor cells.

Mechanism of Action of Cytotoxic Mononuclear Cells and Their Contribution to Host Defense

Cytotoxic killer cells have been implicated in a number of systems of host defense including the destruction of malignant cells. The studies of the types of cells involved, the surface characteristics, cytotoxic repertoire, and the mechanism of action of these various cytotoxic killer cells and their contribution to host defense were continued. Model systems have been developed for the study of killer T cells, cytotoxic K lymphocytes and cytotoxic monocytes in a series of distinct cytotoxic processes. These have included antibody dependent cellular cytotoxicity, mitogen induced cellular cytotoxicity, natural killer and monocyte dependent cytotoxicity and cytotoxicity directed against autologous cells modified by chemicals or viruses.

Over the past year special emphasis continued to be focused on studies of antigen-specific immune responses by human peripheral blood leukocytes in vitro. Influenza viruses have been employed as model antigens for the investigation of cell mediated cytotoxic responses by thymus derived lymphocytes, thymus-dependent lymphocytes (T-cells), and humoral antibody responses by bone-marrow derived lymphocytes (B-cells) whose maturation into immunoglobulin secreting plasma cells is both positively and negatively regulated by T-cells. Also under investigation are the qualities of a third class of mononuclear leukocyte termed monocytes which are required for both T-cell and B-cell responses.

With regard to influenza virus cytotoxic T-lymphocyte (CTL) responses, studies have demonstrated that invdividuals immunized either in vivo or in vitro with viral antigens produce CTL which will lyse homologous antigen bearing autologous (self) target cells but will lyse antigen bearing target cells from other individuals only when the immune CTL and the target cell share genetically determined histocompatibility antigens. The relevant histocompatibility antigens which must be shared between targets and immune CTL are those encoded by a genetic locus termed the major histocompatibility complex (MHC) which in humans codes for the human leukocyte antigens (HLA) A, B, C, DR and D.

Human CTL with specificities for viral antigens and self MHC gene products are produced in vitro by culturing responder peripheral blood mononuclear leukocytes with infectious alloantoic fluid containing influenza A/Hong Kong virus or irradiated influenza virus infected autologous leukocytes for seven days. Such cultures proliferate and generate CTL whose lytic activity is tested on

autologous virus infected targets in a short term ^{51}Cr release assay. Studies were continued to define the cellular requirements for the generation of human influenza virus immune CTL in vitro. Previous studies had demonstrated that T-cells were necessary and sufficient for the generation of self MHC restricted CTL given the provision of adherent "accessory" cells. The maturation of highly purified CTL precursors into CTL effectors capable of target cell lysis was further shown to be dependent on a population of phagocytic adherent monocytes bearing cell surface receptors for the chrystalyzable fragment (Fc) of IgG, and also cell surface molecules recognized by a heteroantisera (anti p 23, 30) directed against the human equivalent of murine immune associated (Ia) antigens. Thus the production of immune CTL was both T-cell and monocyte dependent and non-responsiveness in this assay might be attributable to defects in T-cell and/or monocyte function. These cytotoxic T-cells with receptor specificities for foreign antigen plus autologous MHC antigens are termed self-MHC restricted CTL. Evidence from studies in experimental animals has suggested that self-MHC restricted CTL play a major in vivo role in the recovery from virus infections, immunologic surveillance against neoplasia and deleterious autoimmune phenomena.

Humans with immunodeficiency diseases such as ataxia-telangaiectasia, the Wiskott-Aldrich syndrome and common variable hypogammaglobulinemia (CVH) have recurrent infections, including those caused by viruses, and a greatly increased incidence of neoplasia. Therefore, these patients' lymphocytes were studied for their capacity to generate influenza virus immune CTL. Among ataxia-telangiectasia patients nine of ten studied thus far have failed to generate influenza-specific self MHC restricted CTL as have all seven patients studied with the Wiskott-Aldrich syndrome. In all non-responsive patients this defect was attributable to the lack of effector CTL production since the patients' virally infected target cells could be lysed by immune CTL from HLA related donors. Moreoever, this deficiency could not be explained by reduced proportions of T-cells. In contrast with these results five of five patients studied with CVH and 3 patients with X-linked hypogammaglobulinemia and growth hormone deficiency produced influenza secific self MHC restricted CTL.

These studies demonstrate that in certain patients with hypogammaglobulinemia lacking humoral immune responsiveness to viruses in vivo, CTL effector responses may be retained and contribute to host responsiveness. The inablity to generate CTL in some CVH patients and the majority of ataxia-telangiectasia and Wiskott-Aldrich syndrome patients may contribute to the increased incidence of infections and neoplasia observed in association with these diseases.

In other studies of cell mediated cytotoxicity we have been working on the characterization of the molecular requirements for the expression of synergistic cytotoxicity. This model which measures the ability of human serum factors to activate human monocytes and lymphocytes to kill erythrocyte targets is now substantially characterized. Homogenous purified complement components obtained in collaboration with Dr. Harvey Colton have been used and we have shown that C5 and Factor B in the presence of activated human monocytes (which fail to kill by themselves) are necessary and sufficient to induce lysis. Lymphocytes also exhibit killing in this model and require at least C7 and C8 as well as C5 and factor B. This research is important not only because it represents a

unique model of cell mediated cytotoxicity but also because it unites antigen idependent fluid phase lysis (alternate complement pathway) with antigen independent cell mediated lysis (NK and spontaneous cytotoxicity).

We have also continued our work characterizing the pharmacology of spontaneous monocyte cytotoxicity, a model which measures cell mediated lysis of target cells in the absence of serum factors. Previous work from our laboratory has shown that cytotoxic monocytes are under the control of suppressor cells. We have shown that Cis platinum Adriamycin, X-irradiation and L phenyl alanine mustard all paradoxically enhance monocyte function. Each drug or treatment exhibits unique time constraints and cellular targets. For example x-irradiation enhances killing by inactivation of suppressor cell activity while Cis platinum directly activates monocyte function. These studies have been expanded in clinical studies of cancer patients and early results demonstrate excellent correlation with in vivo and in vitro data. These studies suggest a mechanism resulting in activation of cytotoxic monocytes by agents which are normally thought of as toxic. Such activation may play an important in vivo role. We have also studied anti-inflammatory drugs used in the treatment of rheumatoid arthritis (ASA, Indocin, steroids and gold) and find that these can enhance monocyte function as well.

Mechanism of Action of Growth Hormone

We recently[3] reported that MSA is found in high concentration in fetal rat serum and declined to reach very low levels by day 20 of extrauterine life. This observation led to the proposal that MSA may be a fetal growth factor. Therefore, we were interested in studying the effect of MSA in fetal rat cells in culture. We found that tertiary cultures of rat embryo fibroblasts derived from 16 day embryos have specific receptors for MSA. In contrast to MSA receptors in chick embryo fibroblasts and human skin fibroblasts where insulin competes for ^{125}I-MSA binding, insulin did not compete for ^{125}I-MSA binding to the rat embryo fibroblasts. The rat embryo fibroblasts did not respond to MSA alone (cell multiplication and DNA synthesis) but when added to platelet derived growth factor (PDGF) and platelet poor plasma (PPP) from growth hormone deficient rats (hypox), MSA stimulated DNA synthesis as measured by autoradiography. The combination of MSA, PDGF and hypox PPP produced the same response as a combination of PDGF and PPP from normal rats or normal rat serum. On the other hand, hypox serum or a combination of PDGF and hypox PPP produced only minimal DNA synthesis. Surprisingly, when a 4 day cell multiplication experiment was performed there was no difference in the growth rate of cells maintained in medium containing normal rat serum or hypox rat serum. One explanation for this result would be that the rat embryo fibroblasts were producing MSA and during the 4 day experiment this MSA produced by the rat embryo fibroblasts together with hypox rat serum was as effective as normal rat serum in causing the cells to multiply.

Indeed, when rat embryo fibroblasts were maintained in culture under serum-free conditions and the conditioned medium harvested, MSA was detected in this medium by MSA radioimmunoassay. The concentration of MSA in the medium after 3 days of culture approached the concentration

of MSA in fetal rat serum. Cycloheximide blocked the production of MSA by the rat embryo fibroblasts showing that MSA was actually being synthesized. The MSA produced by the rat embryo fibroblasts was shown to be indistinguishable from MSA produced by the rat liver cell line BRL-3A in radioreceptor assays (chick embryo fibroblasts, chondrosarcoma chondrocytes, and rat liver membranes), a competitive protein binding assay (rat serum binding protein) and bioassay ([^{3}H]thymidine incorporation, chick embryo fibroblasts). Importantly, the rat embryo fibroblast MSA also stimulated the rat embryo fibroblasts to synthesize DNA when added to a combination of PDGF and hypox PPP.

The pygmy (pg/pg) mouse has been proposed as an animal model of growth hormone resistance because treatment of the pygmy mouse with large amounts of growth hormone did not stimulate growth. We showed subsequently that serum somatomedin activity measured by bioassay was normal in the pygmy mouse raising the possibility that the genetic defect in this mouse was distal to somatomedin production, either at the level of the somatomedin receptor or beyond. To investigate this possibility we cultured fibroblasts from skin and kidney of the pygmy mouse and normal littermates. We determined the growth (cell multiplication and [^{3}H]thymidine incorporation into DNA) of the mouse fibroblasts in response to combinations of PDGF, hypox PPP and MSA and also attempted to characterize an MSA receptor with respect to binding affinity for MSA and receptor number. We were not able to detect significant differences between the pygmy and normal fibroblasts for any of these characteristics.

Mouse embryonal teratocarcinoma cells have been shown to differentiate in cell culture to form the many types of tissue making up the teratocarcinoma in animals. Because of the fetal origin of these cells it was of interest to examine the response of these cells to MSA. In collaboration with Wayne Anderson and Lalitha Nagarigan in the Laboratory of Pathophysiology it was found that MSA stimulated the multiplication of F_9/cells, an undifferentiated teratocarcinoma cell line. An MSA receptor was demonstrated on these cells. Insulin was also a mitogen for these cells but did not interact with the MSA receptor suggesting that insulin was stimulating multiplication of the F_9 cells by interacting with an insulin receptor. Earlier studies performed in collaboration with Matthew Rechler, NIAMDD, had demonstrated that insulin-like growth factor receptors were of two types; one which also interacted with insulin and a second which was insulin insensitive. Thus, in a rat liver cell line (BRL 3A-2) ^{125}I-MSA bound to a receptor which was insulin insensitive while ^{125}I-IGF-I bound to a receptor which also interacted with insulin. Recently a collaborative study with Masato Kasuga and Matthew Rechler, NIAMDD, has provided structural evidence for two different types of receptors. Masato Kasya chemically cross-linked ^{125}I-MSA and ^{125}I-IGF-I to receptors on the BRL 3A2 cells and then solulizing the membranes and analyzed the radioligand receptor complexes on SDS disc acrylamide gels. He found that following reduction of disulfide bonds the ^{125}I-MSA receptor complex was a 260,000 dalton species while the insulin sensitive ^{125}I-IGF-I receptor complex was 130,000 daltons.

The Proline Biosynthesis and Metabolism

We propose that the enzymes catalyzing the interconversions of proline, pyrroline-5-carboxylate (PC), ornithine and glutamate constitute a metabolic system for the intercompartmental, intercellular and interorgan transfer of redox potential. Central to this hypothesis is the proline cycle in which proline and pyrroline-5-carboxylic acid (PC) function as a redox pair and their interconversions catalyzed by proline oxidase and pyrroline-5-caraboxylate reductase, mediate the redox transfers. PC reductase, a cytosolic enzyme, oxidizes either NADH or NADPH accompanying the formation of proline. Proline oxidase which is tightly bound to mitochondrial inner membranes converts proline back to PC, donates protons to electron transport and thereby phosphorylates ADP by a mechanism independent of NADH oxidation. The availability of PC for participation in the proline-PC cycle may depend on the enzymes catalyzing the interconversions of ornithine and glutamate, i.e. PC synthase, ornithine aminotransferase and PC dehydrogenase; all of which have PC either as substrate or product and thereby determine the availability of PC for participation in the proline-PC cycle.

We previously showed that the interconversions of proline and PC can regulate $NADP^+$/NADPH redox potential. During the past year we have emphasized 1) the metabolic implications of this regulation and 2) the physiologic interactions of PC-PRO with ornithine and glutamate, precursors of PC.

We showed that in erythrocytes PC not only stimulates the hexosemonophosphate-pentose pathway but also PPRP production and the incorporation of purines into nucleotides by the salvage pathway. In addition, PC regulates an important branchpoint in nucleotide metabolism in that the conversion of adenosine to IMP is stimulated by PC. Thus, through this sequence of metabolic events, PC regulates nucleotide production thereby linking amino acid and nucleic acid metabolism.

The physiologic production of PC from ornithine and glutamate was investigated using fibroblasts from patients with gyrate atrophy. Gyrate atrophy cells lack ornithine aminotransferase and cannot produce PC from ornithine. Added ornithine is toxic to GA cells but not to normal fibroblasts. We showed that the toxicity in gyrate atrophy cells is due to their inability to produce PC. Ornithine is a potent inhibitor of PC synthase which catalyzes the production of PC from glutamate. In gyrate atrophy cells, PC production from glutamate is inhibited by added ornithine and PC production from ornithine is deficient due to the enzyme defect. Normal cells are not sensitive to ornithine toxicity because they can produce PC from ornithine even though the glutamate pathway is inhibited. Thus, ornithine toxicity in gyrate atrophy cells is due to a deficiency in PC production.

Porphyrin Metabolism

Succinylacetone (4,6-dioxoheptanoic acid), an irreversible inhibitor of ALA dehydrase, (the second enzyme of the heme biosynthetic pathway) has been studied for its effects on tumor cells in vitro and in vivo. When administered by intraperitoneally implanted osmotic minipumps, this compound markedly inhibited the growth of the Walker 256 carcinosarcoma and Novikoff hepatoma as ascites tumors. The compound, when started at the time of tumor administration (10^6 cells) and continued for two weeks produced "cure rates" of 100% for these two tumors. However, it had only slight activity in vivo against leukemia L1210 in mice.

In the course of studying the effect of succinylacetone on the growth of the Walker 256 tumor as a solid (subcutaneous) tumor in outbred Sprague Dawley rats, it was shown that tumor graft rejection was prevented by administration of the compound. This demonstration of the inhibition of allograft rejection was followed by two other studies which showed the profound immunosuppressive activity of succinylacetone. First was the fact that human peripheral blood lymphocyte proliferation in response to both mitogens and antigens was markedly inhibited by succinylacetone. Second, administration of the compound to Fischer rats inhibited, by 99% or more, the rise of serum levels of hemolytic antibody to intraperitoneally administered erythrocytes.

Data from our previous studies indicate[1] that the antiproliferative effect of succinyl-acetone results from inhibition of heme biosynthesis in some cells (murine erythroleukemia cells), but operates by a heme independent mechanism in other cells (Walker 256 and Novikoff). Since addition of hematin to the medium did not reverse the inhibition by succinylacetone of human peripheral blood lymphocyte transformation, it is thought that the immunosuppressive effects of the compound operate by a mechanism not related to the inhibition of heme biosynthesis. The immunosuppressive effects of succinylacetone in rats were produced with no gross evidence of toxicity.

The uptake of hematin and porphyrins in normal and malignant cells is being studied. The kinetics of hematin uptake by tumor cells suggest an active facilitated transport mechanism. Uptake by tumor cells can be increased by two separate mechanisms. The first is a gradually increasing capacity (over a period of 3-4 days) for hematin uptake by tumor cells grown in the presence of succinylacetone. The second is a rapidly developing (30 minutes) augmentation of hematin uptake produced by certain local anesthetics and dimethylsulfoxide. These agents also augment hematoporphyrin uptake by tumor cells. The wavelength which is most phototoxic for hematoporphyrin treated tumor cells has been shown to be 503 nm. Phototoxicity of hematoprophyrin treated tumor cells is increased by treatment with certain local anesthetics.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-04020-05-MET

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic Control of the Immune Response to Natural Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.4	3.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms of determinant-specific Ir gene control and of antigen recognition by T and B lymphocytes were explored at several levels in the response to myoglobin. In vitro secondary antibody responses were controlled by the same two determinant-specific Ir genes as the in vivo response, were macrophage and T cell dependent, and seemed to reflect restrictions on T cell-B cell interactions. The antibody response to equine myoglobin, under completely different control from that for sperm whale, required a gene in H-2D, unusual for an antibody response. For staphylococcal nuclease, Ir genes in two high responder haplotypes were found to be nonallelic. Monoclonal antibodies, models of B cell receptors, were found to recognize topographic antigenic determinants, not previously recognized for myoglobin, consisting of residues far apart in the primary sequence but brought together by the folding of the native molecule. Two of these monoclonal antibodies, with different specificities, nevertheless share an idiotope(s) in the combining site. The determinants recognized by some T cell populations have been similarly identified. Both T cells and antibodies are surprisingly sensitive to Glu --> Asp substitutions.

Project Description:

Objectives: The critical importance of genetic factors in regulating the immune response, and especially of genes which appear to be specific for particular antigens (so-called immune response or Ir genes), has become increasingly apparent in the last 15 years. However, the mechanism of action of these genes and the nature of the product encoded by them remain unknown. Most of the Ir genes so far described are linked to the major transplantation or histocompatibility gene complex. The primary objective of this project has been to define the mechanism by which Ir genes linked to these major transplantation antigens (HLA in man and H-2 in the mouse) control the immune response to natural protein antigens, with the hope of understanding the modes of recognition involved at this regulatory level, the cells which participate and the cell membrane receptors which mediate the regulation.

Methods Employed: Myoglobins from various species were purified by the method of Hapner et al., (J. Biol. Chem. 243:683 (1968)). Fragments of myoglobin were prepared by CNBr cleavage and chromatographic purification. For antibody responses, mice were immunized i.p. with 200 micrograms in complete Freund's adjuvant and boosted twice with 100 micrograms in saline, as described previously
(Berzofsky, J. Immunol. 120:360 (1978). Myoglobin and its fragments were radiolabeled at the N-terminal alpha-amino group with $K^{14}CNO$ or N-succinimidyl-[2,3-^{3}H] propionate. Antibodies to these were measured using the polyethylene glycol precipitation direct binding assay described previously (Berzofsky, J.A., J.Immunol., 120:360 (1978)). In competition studies, other unlabeled myoglobins were added at various concentrations.

T-lymphocyte proliferation was studied by a method based on that of Corradin, Etlinger and Chiller (J. Immunol. 119, 1048 (1977). Briefly, nylon-wool passed T-cells from draining lymph nodes of mice immunized s.c. in the tail 8 days previously with myoglobin in Freund's adjuvant were cultured for 5 days in the presence of varying concentrations of antigen (myoglobin or fragments). Proliferation was assessed from the amount of methyl-^{3}H-thymidine incorporated into DNA during the final 4 hours of culture. For experiments assessing macrophage reconstitution of response, such T cells were passed twice over nylon wool to deplete of macrophages. For a source of macrophages, a method was developed for purifying liver macrophages (Kupffer cells) by perfusion in situ, triteration, treatment with collagenase and DNase to free the macrophages, density gradient centrifugation over metrizamide, glass adherence, and irradiation with 1500 R from ^{137}Cs before use. These were cultured with macrophage depleted T cells and antigen as described above, or were pretreated with antigen in culture medium at 37° in polypropylene tubes at 10^{6} cells/ 0.2 ml for one to 24 hours, washed and then added to macrophage-depleted T cells without soluble antigen present during the 5-day culture, to assess stimulation of the T cells. In experiments to determine the effect of anti-Ia antibodies, dilutions of appropriate antisera or monoclonal hybridoma culture fluids were added during the pretreatment of the liver macrophages with antigen, and then washed out before these cells were mixed with T cells.

A culture system for assessment of in vitro antibody responses to myoglobin and its fragments was developed by a modification of that of Mishell and Dutton (J. Exp. Med. 126: 423 (1967)). Optimum conditions were determined to be as follows: 4 X 10^6 spleen cells from immunized mice were cultured with 0.1 to 1.0 ug myoglobin in 1.5 ml of supplemented RPMI-1640 medium with 10% fetal calf serum in flat-bottom wells for 9 days at 37°, 6% CO_2, on a rocking platform. On the fourth day one ml of supernatant was exchanged for fresh medium, and cultures were fed daily thereafter. On the 9th day, culture supernatants were taken to test for the presence of secreted antibody, or else cells were harvested on day 6 to count antibody-secreting cells by the hemolytic plaque method. Besides whole spleen, various cell mixtures could be cultured. For instance, spleen cells depleted of macrophages by passage over Sephadex G10 (Hodes and Singer, Eur. J. Immunol. 7: 892 (1977)) were cultured alone or with macrophages from syngeneic or semisyngeneic sources. Macrophages were either liver Kupffer cells (described above), or irradiated, glass-adherent, splenic macrophages. T cells were eliminated, where necessary, by treatment with rabbit anti-mouse brain antiserum plus complement. T cells were purified, where necessary, by removal of B cells and macrophages on nylon wool columns.

Concentrations of antibodies in culture supernatants were measured by a solid phase radiobinding assay. Wells of polyvinyl chloride flexible microtiter plates were coated by incubation with 50 ul myoglobin, 100 ug/ml, for 1.5 hrs. Unoccupied sites on the well were saturated by a 15 minute incubation with 10% bovine serum albumin. Then 50 ul aliquots of culture supernatants were incubated in the wells for 1.5 hours and unbound material washed out. Finally, ^3H-labeled, affinity purified, goat antibody to the Fab fragment of mouse IgG was incubated in the wells for 1.5 hrs to bind to any antibody attached to the myoglobin on the plastic. All incubations were carried out at room temperature, and were followed by 3 washes with 1% bovine serum albumin in saline. Individual wells of the plate were the cut apart and radioactivity determined by scintillation counting. Controls for nonspecific binding were all close to machine background. A standard curve with known antimyoglobin antibody was used for quantitation.

Hybridoma monoclonal antibodies specific for sperm whale myoglobin were described in the previous annual report (1980-81). These had high affinities, between 0.2 X 10^9 and 2.2 X 10^9 M^{-1}. Their relative affinities for myoglobins of other species were determined by competitive radioimmunoassays, in which increasing concentrations of competitor were added to a constant concentration of labeled sperm whale myoglobin (7 nM) and of monoclonal antibody (on the order of 4-6 nM), and polyethylene glycol (MW 6000, final concentration 10%) used to precipitate all immunoglobulin plus bound antigen. The concentration of competitor resulting in a 50% decrease in bound/free ratio for labeled myoglobin was taken as a measure of relative dissociation constant.

Antiidiotypic antibodies were raised in guinea pigs, tolerized one day previously with 5 mg of diaggregated mouse Ig given intravenously, by immunizing subcutaneously in the footpads with 20 micrograms of affinity purified monoclonal antibody emulsified in complete Freunds adjuvant. The animals were boosted intramuscularly three and five weeks later, first with 20 micrograms in complete adjuvant, and bled repeatedly from 10-18 days after the last immunization. The sera were absorbed extensively by repeated passage through columns of normal mouse Ig-Sepharose to remove antibodies to constant region determinants. They were studies using an enzyme-linked immunoassay (Elisa) as follows: Myoglobin was bound to polyvinyl chloride microtiter plates. Alkaline-phosphatase conjugated monoclonal antimyoglobin antibodies were preincubated with a putative antidiotypic antisera, and then incubated on the myoglobin-coated plastic. After washing to remove unbound material, the amount of enzyme bound was determined by optical absorbance change at 405 nm after addition of p-nitrophenyl phosphate as substrate.

Major Findings:

Background: We have previously studied the in vivo serum antibody response and in vitro T-lymphocyte proliferative response to a natural protein antigen, sperm whale myoglobin (Mb), and shown that both of these responses are controlled by the same Ir genes (Berzofsky, J. A., J. Immunol. 120: 360 (1978)). Using inbred strains of mice which differ only in the H-2 complex (known as congenic strains), we demonstrated that two different genes, mapping in distinct subregions (I-A and I-C) of the I region of H-2, controlled the responses to different antigenic determinants (distinct chemical sites) on the same antigen molecule - the first example of such independent control of responses to different chemical moieties on the same protein molecule. Moreover, the gene which controlled the T-cell proliferative response to a given determinant of myoglobin also controlled the production of antibodies specific for that same region of the molecule - an indication of parallel control of T-lymphocyte recognition and activation of B lymphocytes with similar specificity (Berzofsky et al.: Proc. Natl. Acad. Sci 76:4046 (1979).

One level at which this Ir-gene-controlled choice of antigenic determinants recognizable is mediated is the recognition by T-lymphocytes of antigen in association with cell surface structures on macrophages, which serve as antigen presenting cells. These surface structures, which differ in different inbred strains of mice, are encoded by genes also mapping in the I region of H-2 , and are therefore designated Ia (for I-associated) antigens. In our experiments, myoglobin-immune lymph node T cells, depleted of macrophages, from (high responder X low responder) F_1 hybrid mice were held constant as the source of responding (proliferating) T cells, and the cultures were reconstituted with liver macrophages from high responder parental mice with both Ir genes, low responder parental with neither gene, or recombinant mice with only one gene or the other. We found that the magnitude of response, and the selection of which fragments of myoglobin could stimulate these F_1 T cells in vitro was determined by the source of the macrophages, and corresponded exactly to the response pattern of the strain of mice from which the macrophages

were obtained (Richman et al.: J. Immunol. 124:619 (1980). Macrophage pretreated with antigen could also stimulate immune T cells in the absence of soluble antigen. This stimulation manifested the same Ir genetic restriction for macrophage source, and could be inhibited by inclusion of appropriate monoclonal or conventional anti-Ia antibodies, without complement, during the pretreatment with antigen.

Current Results:

The limitation of T cell proliferative responses is that they involve only T cells and macrophages, not B lymphocytes which produce antibody. In order to study the mechanisms of determinant-specific Ir gene control of the antibody response to myoglobin in vitro, we have now developed a modified Mishell-Dutton culture system in which secreted antibody specific for myoglobin can be measured in the culture supernatants by a solid phase radioimmunoassay. By this method, we can also measure the fine specificity of the supernatnat antibodies for different fragments of myoglobin. The in vitro spleen cell antibody response to sperm whale myoglobin was found to be controlled by the same Ir genes which controlled the in vivo response, at the level of individual determinants on different fragments of myoglobin. In addition, we have extended the mapping to recombinant H-2 haplotypes not previously studied. For instance, the $H\text{-}2^d$ high responder haplotype has two Ir genes for myoglobin, one in I-A and one in I-C. Recombinant strains with the $H\text{-}2^d$ allele only in I-C can make antibodies which bind to fragment (1-55) of myoglobin but not antibodies specific for fragment (132-153). Recombinant strains with the $H\text{-}2^d$ allele only in I-A make antimyoglobin antibodies that react with both fragments. In contrast, the high response $H\text{-}2^s$ haplotype has only one Ir gene we can detect for myoglobin, mapping in I-A (or I-B). The recominant strain B10.S(9R), which has the $I\text{-}C^d$ gene in addition to the $I\text{-}A^s$ gene, makes more antibody to whole myoglobin and fragment (1-55) then the B10.HTT strain, which has only the latter gene. In contrast, both strains make comparable amounts of antibody binding to fragment (132-153), since the additional gene in $I\text{-}C^d$ does not contribute to this component of the response. The non-H-2-linked Ir gene which caused a greater overall response in congenic strains on the A background compared to those on the B10 background operated in vitro as it had in vivo - an indication that the mechanism did not involve clearance of antigen or antibody in the whole organism.

We have found this in vitro antibody response to be macrophage and helper T-cell dependent, as shown by experiments depleting these cells, and reconstituting with purified T cell or macrophage populations. In order to dissect the cell interactions involved in mediating this Ir gene control, we have mixed cell populations from different strains, in combinations which would not produce an allogeneic effect. Myoglobin-immune helper T cells from (high responder x low responder) F_1 hybrid mice would help T cell-depleted populations of B cells plus macrophages from myoglobin immune high responder mice but not myoglobin immune low responder mice. To be sure that the low responder B cells were adequately primed in vivo, we must use B cells from mice immunized with myoglobin coupled to an immunogenic carrier. To distinguish between a defect in T-cell-B cell interaction and a defect in T-cell macrophage interaction, we must add F_1 macrophages to the low responder B cells to see

if these overcome the defect. However, when we did the reciprocal experiment of depleting macrophages alone from the F_1 spleen cell populations of T and B cells, macrophages of either the high responder parent or low responder parent could equally well reconstitute the response. This result was also found when the macrophages were pretreated with myoglobin and then no additional soluble antigen added. Thus, while we cannot exclude the possibility that residual F_1 macrophages are presenting antigen, we have been unable to demonstrate an Ir genetic restriction on macrophage function in this in vitro secondary antibody response, despite multiple attempts. Therefore, since we did observe a Ir restriction for B cells plus macrophages, it is possible that in the in vitro secondary antibody response to myoglobin, Ir gene function is involved in T cell-B cell interactions but not in macrophage-lymphocyte interactions. Further tests of this possibility, as well as studies of T cell function (using T cells from neonatally tolerized mice to avoid allogeneic effects), are in progress.

In order to identify the structural features of sperm whale myoglobin that make it immunogenic in high responders and nonimmunogenic in low responders, we studied the Ir gene control of the response to other myoglobins. The antibody response to equine myoglobin, which shares the three-dimensional structure and 87% of the sequence of sperm whale myoglobin, was found to be under completely different Ir gene control from that to sperm whale myoglobin. Mice (such as B10.BR) that were low responders to sperm whale myoglobin were high responders to equine myoglobin. Moreover, the Ir genes mapped in different loci. The antibody response to equine myoglobin was controlled by complementing Ir genes in I-A and H-2D. We were surprised to find an Ir gene for an antibody response to a soluble protein antigen mapping in H-2D, rather than the I region, since none had mapped there before. Thus, either the mechanism of action of this Ir gene is different from those in the I region, or equine myoglobin is the first soluble protein antigen recognized in association with a class I (H-2K/D) rather than Ia antigen. These alternatives are under study. Also, by studying the responses to myoglobins with sequences between those of sperm whale and horse, we hope to localize th antigenic determinants recognized by the different Ir genes.

In a related study of the antibody response to staphylococcal nuclease in collaboration with David H. Sachs (Immunology Branch, NCI), we found that the high responder gene in the $H\text{-}2^d$ haplotype maps in I-A, whereas that in the $H\text{-}2^k$ haplotype maps in I-B. Therefore, high responder Ir genes for the same antigen in different haplotypes need not be allelic.

To understand the molecular mechanism of this Ir gene control, one would like to understand the nature of the receptors for myoglobin on B cells and T cells. As a model of the former, we raised monoclonal hybridoma antibodies against sperm whale myoglobin. Although these had a high affinity ($10^9 M^{-1}$) for native myoglobin, all six monoclonal antibodies studied in detail failed to bind to any of the three CNBr cleavage fragments which together span the whole sequence of myoglobin. Therefore, they must have been highly specific for the native conformation. Since

we could not use fragments to define the antigenic determinants recognized by these monoclonal antibodies, we used a series of 15 native myoglobins of similar three-dimensional structure and known amino acid sequence differences. By correlating the relative affinity, measured by competitive radioimmunoassay, with the amino acid substitutions, we were able to identify critical residues recognized by three of the monoclonal antibodies. Two of these recognized clusters of residues on the surface of myoglobin which were far apart in the primary sequence but brought together by the folding of the native molecule. These, termed topographic determinants, accounted for the failure to bind random conformation fragments of myoglobin. Monoclonal antibody of clone 3.4 binds a determinant involving Glu 4, Lys 79, and probably His 12. Glu 4 and Lys 79, although far apart in the sequence, are brought so close on the surface of the native molecule that they form a tight ionic bond. When the Glu is replaced by an Asp, although the functional group is the same, the Asp cannot reach as close to Lys 79, so that the two charged groups are less neutralized, and the antibody binds with a much lower affinity. A second example is the antibody of clone 1, which binds a determinant involving Glu 83 on the E-F interhelical segment and Ala 144 and Lys 145 on the H helix. These residues also form a topographic determinant created by the folding of the native molecule. The third monoclonal antibody of clone 5 distinguishes exquisitely between those myoglobins with Lys 140 and those with an asparagine substitution at this site. Although we have not identified other residues in this antigenic determinant, it is presumably also specific to the native conformation, since this antibody does not bind fragment (132-153) containing residue 140. Interestingly, the residues identified for all three antibodies were largely outside the previously reported sequential determinants of myoglobin. The monoclonal antibodies were sensitive to subtle changes within antigenic determinants, such as the replacement of glutamic with aspartic acid. We are now attempting to sequence the variable regions of these antibodies to understand the structure of the antibody combining sites complementary to these determinants of myoglobin.

We have also raised guinea pig anti-idiotypic antibodies against these monoclonal antibodies. Although no idiotype common to serum anti-myoglobin antibodies was found, we discovered an intriguing crossreaction between the idiotypes of two monoclonal antibodies with different specificity. Guinea pig antibodies specific for clone 4 antibodies inhibited the binding to myoglobin of antibodies of clone 1 as well as clone 4, but not clones 5 or 6. All 4 monoclonal antibodies were IgG_1, kappa, but had different fine specificity. Similarly, antiidiotypic antibodies to clone 5 monoclonal antimyoglobin inhibited the binding of antibodies from clones 1 and 4, as well as clone 5, but not clone 6. In the case of antiidiotypic antibodies to clone 4 antimyoglobin which crossreacted with clone 1 antimyoglobin, the shared idiotope(s) was within or very near the combining site, in that myoglobin could inhibit (by 80%) the crossreactive idiotype - antiidiotype interaction. Why should monoclonal antibodies recognizing different determinants of myoglobin share part of their combining sites? Perhaps a common antiidiotypic helper T cell was responsible for promoting these clones, which both arose in the same spleen.

To study the antigenic determinants of myoglobin recognized by T lymphocytes, we used an approach similar to that used for the monoclonal antibodies. Mice were immunized subcutaneously with myoglobin of one species, and their draining lymph node cells were tested for a proliferative response in vitro to myoglobins of other species. Assuming that those myoglobins which stimulated shared one or more determinants with the immunizing myoglobin, while those which failed to stimulate shared none, we were able to localize antigenic residues in several cases. B10.S T cells appear to be highly sensitive to the difference between glutamic acid (Glu) and aspartic acid (Asp) at position 109. If immunized with sperm whale myoglobin (Glu 109), they responded only to myoglobins bearing Glu, not Asp, at 109. In contrast, when immunized with equine myoglobin (Asp 109), the reverse was true. Thus, noncrossreacting populations of T cells could be elicited in the same mouse, each specific for a carboxylic acid residue at position 109, but able to distinguish between the Glu and the Asp, which differ by 1 carbon atom in side chain length. Since the same macrophages can present both myoglobins (and since the B10.S has only an I-A molecule but appears to lack an I-E molecule), we believe this discriminatory ability is a function of the T cell receptor, not the macrophage. In another strain (B10.GD), the Glu 109 of sperm whale myoglobin also appears to be critical, but, in addition, a change at position 116 (histidine ---> glutamine) also interferes with T cell stimulation. Thus, this strain appears to recognize a determinant overlapping with but distinct from that recognized by the B10.S strain. Moreover, the two residues involved are 7 residues apart in the primary sequence, but are brought together by the folding of the native molecule. While these residues are close enough to be on the same fragment of myoglobin, they may still represent a topographic determinant recognized by T cells.

In order to extend this analysis as well as to isolate the receptors involved, we are currently trying to prepare monoclonal populations of T cells. We would like not only to compare the receptors of T cells and B cells for myoglobin, but also to analyze the role each cell plays in the genetic control of the immune response.

Significance to Biomedical Research: The development of an in vitro culture system in which we can measure secreted antibody, rather than plaque-forming cells, in a secondary immune response, allows one to determine quantity, fine specificity, subclass, allotype, idiotype, and any other property which we might try to test later, all on the same population of antibodies. The supernatants can be frozen for retesting or new assays at a later date, in contrast to plaques, which are lost on assay. Thus, we can explore the role of T-cell-B-cell, T-cell-macrphage, and even B cell-macrophage interactions in the mechanism of Ir gene control which yet remains to be elucidated. Our results so far indicate that not all of the control may occur at the T-cell-macrophage interaction, as some have suggested. The Ir gene controlling the response to equine myoglobin which maps in H-2D is unprecedented. It suggests either a new type of Ir gene, or a new type of genetic restriction element for presentation of soluble antigen. The nonallelic nature of high responder Ir genes for staphylococcal nuclease in different H-2 haplotypes implies that different genes can subserve the same function, and cautions against

the unfortunate but not uncommon practice of mixing data from different high responder haplotypes in the mapping of an Ir gene. The topographic antigenic determinants defined on myoglobin demonstrate a new type of determinant not previously recognized on myoglobin, and emphasize the importance of the surface topography and conformation for antigenicity. The fine discriminating ability of these monoclonal antibodies for subtle changes in the antigenic determinant would not have been possible with conventional antisera. The sequencing of these monoclonal antibodies should allow us to define the nature of the interaction between globular protein antigen and globular protein antibody in a way not possible with myeloma proteins, which bound haptens. The antiidiotype studies address not only the relationship between idiotype and combining site structure and specificity, but also the network regulation of induction or repression of particular idiotypes. Finally, the studies of T cell receptors and specificity should help elucidate the relationship between the specificity of T cells, of B cells, and of the Ir genes themselves. Moreover, if topographic determinants requiring the native structure can be demonstrated to be recognized by T cells, this would imply that macrophage processing involving cleavage of antigen into fragments is not an obligatory step in T cell recognition.

All of these studies are hoped to ultimately shed light on the mechanisms and role of genetic controls on immune responses which play a role in human disease, whether they be normal host defenses, excessive responses which escape control (in autoimmune disease), or deficient responses, as in malignancy or immunodeficiency diseases.

Proposed Course: Further studies of the cell interactions mediating the Ir gene control of the in vitro antibody response are in progress. In addition to the proposed experiments already mentioned above, we are preparing neonatally tolerized mice so that the ability of parental high and low responder T cells can be studied with F_1 B cells and macrophages without a complicating allogeneic effect. We are also trying to study the effect of helper T cells specific for particular determinants on the selection of B cells with specificity for the same or different determinants, since we have already demonstrated that the carrier effect does not extend through the whole molecule. We are examning the H-2D linked gene controlling the response to equine myoglobin using mutant mice with mutations at this locus, and using anti-H-2D antibodies as blocking agents to see whether this gene also controls antigen presentation. This question can also be approached using F_1 T cells and parental macrophages. The monoclonal antibody study is being pursued in several directions. We are studying the effect of pH on affinity to examine the role of electrostatic interactions on antigen-antibody binding, in collaboration with Professor Frank R.N. Gurd and coworkers (Indiana University). The binding sites are being further explored using semisynthetic myoglobins prepared by the Indiana group. We plan to study the kinetics of this iteraction in collaboration with Dr. Robert Noble (SUNY at Buffalo). We are preparing isolated chains to sequence in collaboration with Dr. Stuart Rudikoff (NIH), and Fab fragments to try to crystallize for x-ray diffraction studies of the three-dimensional structure of the antibody combining site in collaboration with Dr. David Davies (NIH). We are also attempting

to prepare monoclonal antiidiotypic antibodies, and to look for these idiotypes in immune serum. In the T cell proliferative response we are further defining the sites recognized by T cells, and using a similar approach to study the determinants responsible for Ir gene controlled immunogenicity of different myoglobins - the closest thing to the specificity of the Ir gene itself. Finally, we are attempting to prepare isolated T cell clones to study their specificity and their function in the antibody response and the control of antibody specificity and to isolate and characterize their receptors.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-04019-08-MET

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunoregulatory Cells in Human Neoplasia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Jack Strominger, M.D.	Professor of Biochemistry	Harvard Medical School
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COOPERATING UNITS (if any)

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

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NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to analyze the cellular control of immune function in normal individuals and in certain patients with neoplastic disease. We are currently determining the helper and suppressor activity of neoplastic T cells from patients with acute lymphoblastic leukemia and cutaneous T-cell lymphomas and also the capacity to induce differentiation in these cells. We are also studying abnormalities of regulatory monocytes and T-cells from patients with multiple myeloma and mice bearing plasmacytomas. We are characterizing the capacity of antibodies directed against surface membrane products of immune response genes to inhibit human immune reactions by activating regulatory T cells. We have analyzed the expression of T-cell Fc receptors and the role of cytoskeletal structures in normal Fc expression.

PHS-6040
(Rev. 2-81)

Objectives: T cells play a critical role in a number of immune systems. These cells are involved in cell-mediated immunity which includes such phenomena as delayed hypersensitivity, allograft rejections, graft vs. host reaction, tumor cell killing in some systems, and lysis of virally-infected target cells. Normal T cells proliferate in vitro in the presence of plant mitogens, such as phytohemagglutinin and concanavalin A. They may mediate the mixed-lymphocyte-reaction, act as killer cells in certain models of lymphocyte-induced cytotoxicity, and generate soluble effector proteins such as lymphokines. In addition, T cells play an indespensible role in regulating humoral immune response by acting as potentiators (helper cells) or inhibitors (suppressor cells) of the transition of B cells into immunoglobulin producing plasma cells. Both antigen-specific and nonspecific helper and suppressor functions have been identified.

In this project, we have examined regulatory T cells in four biologic settings. The first setting relates to the observation that certain T-cell lines - - and neoplastic T cells from certain patients with leukemias or lymphomas may still retain immunoregulatory properties. The second setting relates to the observation that host T cells and macrophages from mice bearing plasmacytomas (which greatly resemble the human cancer multiple myeloma) may play a role in the generalized humoral immunodeficiency state associated with plasma-cell tumors. The third setting relates to the observation that antibodies directed against human Ia (immune response associated) antigens can depress humoral immune function by activating a radiosensitive suppressor T-cell population. The fourth setting relates to T cells that express different Fc receptors. We believe these four areas of investigation have both clnical and theoretical ramifications.

Methods Employed: To study the transition of circulating human lymphocytes into immunoglobulin secreting plasma cells, peripheral lymphocytes were cultured in the presence of pokeweed mitogen, a water soluble mitogen derived from a Nocardia opaca, and Epstein-Barr virus. The amount of IgM, IgA and IgG synthesized and secreted into the media was determined by double-antibody radioimmunoassays or by the reverse hemolytic plaque assay. The presence of circulating suppressor cells was assayed by a co-culture technique. Circulating neoplastic T cells (and T-lines) from various patients and peripheral blood indicator lymphocytes from normal control individuals were co-cultured. The synthesis of immunoglobulin by cells of the two subjects in the co-culture was related to the sum of the expected contribution of each population. In order to assay helper activity purified B cell populations, freed of T cells, were used as indicator cells in the presence of pokeweed mitogen. It should be noted that pokeweed mitogen is a highly T-cell dependent activator, whereas Epstein-Barr virus (EBV) and Nocardia opaca water soluble mitogen are relatively thymic independent polyclonal activators, and this will be discussed further below.

We have established and are now carrying both cloned and uncloned T-cell lines, which are T-cell growth factor dependent. Certain cell-surface-membrane markers were analyzed using monoclonal antibodies in an indirect fluorescence activated cell sorter (FACS) system. We analyzed

T-cell receptors for the Fc piece of IgM and IgG by sensitized ox-red-cell rosette formation.

Primary sheep red cell specific immune responses of murine spleen cells were assessed by the standard in vitro Mishell-Dutton culture systems and Jerne plaque formation.

To study cell-membrane antigen-synthesis cells were pulsed with ^{35}S-methionine (in collaboration with Dr. Strominger and his co-workers). Solubilized membrane proteins were subjected to sodium dodecyl sulfate polyacrylamide gel analysis using 7-15% gradients (in the presence of 2-mercaptoethanol). Labelled proteins were precipitated using rabbit antibodies and protein A from Staphylococcus aureus.

NEOPLASMS OF REGULATORY CELLS

Background: A number of essential insights into the nature of the humoral immune responses resulted from the study of neoplasms of the B cell/plasma cell series. The recognition that so-called paraproteins derived from patients and certain animals with multiple myeloma (a malignant proliferation of plasma cells) represented extremely homogeneous immunoglobulins has affected essentially every phase of immunologic research. An understanding of antibody structure, function, metabolism and genetics depends heavily on the use of such homogeneous immunoglobins. Indeed, certain immunoglobulin classes (such as IgD and IgE) would have been difficult or impossible to evaluate without the availability of myeloma-related proteins. Recently recognized membrane and functional differences between B cells and T cells provided an important new basis for classifying neoplastic lymphocytes. Such a classification has already proved to have therapeutic and prognostic relevance in characterizing lymphomas and certain leukemias. The study of malignant T cells and their products may prove to be as important in answering questions regarding cellular immunity, and especially, the T-cell regulation of humoral immune responses, as myeloma cells and their protein products have proved to be in answering questions concerning the immunoglobulin synthesizing system. We have focused a considerable degree of research and clinical effort in studying patients with acute and chronic leukemias thought to be of T-cell origin. During the past year we have extended our analysis of the immunoregulatory properties of leukemic T cells from patients with Sezary syndrome*, and we have given special attention to the leukemic T cells of some patients with childhood lymphoblastic leukemia. We have previously shown that certain patients with the Sezary syndrome may have leukemic cells which act as so-called helper T cells. We have observed a helper-like activity in

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*The Sezary syndrome is a cutaneous T-cell lymphoma in adults characterized by severe erythroderma, diffuse lymphadenopathy, and circulating neoplastic cells with cerebriform nuclei. Mycosis fungoides is a related aleukemic cutaneous T-cell lymphoma.

leukemic cells from one patient with acute T-cell leukemia, and in one clone of cells from a patient with mycosis fungoides. By refining our analysis, we have learned that approximately one-half of patients studied had helper activity in a system where any contaminating residual normal T-cell effects were minimized by dilution studies. We have discovered one patient with acute lymphoblastic leukemia whose neoplastic cells acted as T cells that could perform a helper function. Also, we have analyzed the neoplastic cells from an unusual child with a cutaneous T-lymphoma, and found them to function as potent suppressor cells. Furthermore, we found another patient with acute lymphoblastic leukemia (also T-cell type) who had hypogammaglobulinemia prior to chemotherapy, and whose neoplastic cells appeared to function as pro-suppressor cells. Such cells required T-T interaction for maximal suppressor effect. Antibodies raised against these suppressor-like leukemic T-cells appear to react with surface membrane glycoproteins found on certain normal cultured cells with suppressor properties.

Major Findings: The neoplastic lymphocytes of essentially all patients with Sezary type T-cell leukemias failed to produce immunoglobulin under any of the conditions assayed. None of the Sezary patients studied had neoplastic T cells which exhibited suppressor cell activity using pokeweed mitogen. Indeed, on a number of occasions, normal lymphocytes showed a greatly amplified immunoglobulin synthesizing and secreting capacity in the presence of Sezary cells.

We have data supporting the view that some (but not all) patients with the Sezary syndrome variant of cutaneous T-cell lymphoma have a disease that represents a homogeneous expansion of polyclonally active helper-like T cells. Highly purified normal B cells do not undergo a transition into immunoglobulin-secreting cells in vitro after stimulation with certain lectins, such as pokweeed mitogen, unless a source of helper T cells is provided. The neoplastic T cells from certain patients with the Sezary syndrome provide such a helper effect by promoting the maturation of normal indicator B cells (rigorously depleted of T cells) into immunoglobulin-secreting cells following stimulation with pokeweed mitogen in vitro.

We have examined the surface membrane phenotype of Sezary cells using certain monoclonal reagents. Essentially all Sezary T cells studied express the OKT4 antigen (thought to be present on normal helper T cells), but did not express the OKT8 antigen (thought to be present on normal suppressor T cells). We consider these membrane-marker studies as supplements to functional studies. At this time, we do not believe that membrane-marker studies alone can be used to ascribe immunoregulatory characteristics to neoplastic cells. We have succeeded in establishing both uncloned and cloned T-cell lines from peripheral blood samples. The surface antigenic phenotypes did not correlate with regulatory function in certain cases. In addition, further research is needed to define the stability of these markers.

We are continuing our research efforts to learn whether neoplastic T cells with idiotype-specific helper activity exist, and also whether normal T cells augment or modify some neoplastic helper effects. Certain Sezary cell populations seem to have aberrant immunoglobulin gene rearrangements. How these affect their regulatory capacity is not known. The full range of immunoregulatory characteristics of Sezary cells is a subject for further research. We are actively seeking to produce somatic cell hybrids between functional Sezary cells and appropriate long term (purine salvage pathway deficient) T-cell lines as a potential resource for studying regulatory cell function.

NEOPLASTIC PRO-SUPPRESSOR CELLS FROM A CHILD WITH ACUTE LYMPHOBLASTIC LEUKEMIA AND HYPOGAMMAGLOBULINEMIA

We recently investigated an infant boy with acute lymphoblastic leukemia who had hypogammglobulinemia at initial presentation. The neoblastic cells were of T-cell origin and had an unexpectedly low terminal transferase activity. One of the most striking features of this child's unusual disease was a profound depression of serum IgM and IgG concentrations. The child received successful multimodal inductive therapy for acute lymphoblastic leukemia, after which the serum immunoglobulin levels temporarily rose. During the preterminal relapse of the leukemia, there was once again a depression of serum IgM and IgG levels.

Our studies of this patient indicated that the leukemic cells had a potent suppressor effect under suitable conditions of pokeweed mitogen activation. However, the leukemic cells could not bring about a usppressor effect without the cooperation of a radiosensitive subset of normal T cells. In more recent experiments we found that these leukemic T cells appear to function as a population of pro-suppressor T cells which can mature into suppressor effector T cells following interaction with a population of normal suppressor activator or inducer T cells. This maturation did not occur when another polyclonal activator (Nocardia water-soluble mitogen) was used unless pokeweed mitogen was also provided. In some settings the neoplastic pro-suppressor T cells could mature into fully functional suppresor effecor T cells under the influence of a soluble factor secreted by pokeweed mitogen-triggered cooperating normal T cells. In the process of maturing into functional suppressor effectors, the leukemic cells began to display the OKT3 antigen and the Tac antigenic marker. The Tac antigen is a large molecular weight (120,000 daltons) protein normally expressed on activated regulatory T cells with effector function (but not on resting, precursor T cells). Tac antigen is not detected on B cells or monocytes. This provides evidence that leukemic regulatory cells may still acquire new antigens and new functional capacity under the influence of normal T-cell inducing factors.

In other studies we found that these pro-suppressor leukemic suppessor cells could be used to raise antibodies that react with a set of very high-molecular weight glycoproteins (different from the Tac antigen) found on the surface of certain cultured normal T cells exhibiting an in vitro suppressor activity for immunoglobulin production. These glycoproteins have a molecular weight of approximately 200,000 daltons -- and are rich in mannose residues.

Based on our studies with these neoplastic pro-suppressor T cells, we have constructed a general hypothesis for the induction of human suppressor effector cells. In this hypothesis, human suppressor T cells exist for a variable time as pro-suppressor cells. At this stage of maturation, such cells do not have significant immunoregulatory capacity. Under the proper circumstances pro-suppressor T cells may interact with at least one other set of activating T cells, or soluble factors derived from these cells. It is likely that the pro-suppressor T cells must be capable of synthesizing DNA de novo in order to mediate suppression fully. Once the pro-suppressor T-cell subset has differentiated into a suppressor effector T-cell subset, further DNA synthesis is probably no longer necessary for suppressor function. In this process of differentiation, regulatory T cells display the Tac antigen.

REGULATORY CELL ABNORMALITIES IN THE HUMORAL IMMUNODEFICIENCY ASSOCIATED WITH PLASMA CELL TUMORS

Background: A long-standing research interest within the Metabolism Branch has been the depression of immune function seen in patients with multiple myeloma. As already touched upon, multiple myeloma is a disease that represents the monoclonal expansion of malignant plasma cells, which generally secrete a homogeneous immunoglobulin (or immunoglobulin subunit) product. Such products are termed paraproteins. Patients with multiple myeloma demonstrate impaired antibody formation after antigenic stimulation, have depressed polyclonal (non-paraprotein) immunoglobulin levels in their serum, and are readily susceptible to infection with highly pathogeneic bacteria. Metabolic turnover studies performed within the Branch have demonstrated that this depression of polyclonal immunoglobulins is related to an increased plasma volume or increased fractional catabolic rate in special circumstances. However, the same studies have shown that the most important factor contributing to the depressed serum levels of non-paraprotein immunoglobulins is a decreased in vivo synthetic rate. Certain strains of mice (especially Balb/C) given appropriate inflammation - inducing treatments may acquire malignant plasmacytomas which are associated with several key features of human multiple myeloma. Such plasmacytomas are often transplantable into syngeneic normal mice and the transplantable tumors bring about a form of humoral immunosuppression in recipient animals which is quite analogous to the depressed immunity seen in patients with multiple myeloma.

Major Findings: We previously observed that a significant number of patients with multiple myeloma have circulating host regulatory cell abnormalities. We found that macrophage-like cells from certain patients with multiple myeloma can function as potent inhibitors of immune function. Removal of such macrophage-like suppressor cells in vitro nullified the excess suppressor function seen in such patients, and, in fact, could partially restore the immune competence of the patient-circulating lymphocyte populations. Using patients with multiple myeloma, it has thus far been very difficult to define whether abnormal regulatory T cells also play a role in the humoral immune impairment.

In collaboration with Dr. Linda Muul, we have attempted to further elucidate the regulatory abnormalities seen in myeloma by using Balb/C mice carrying transplantable plasmacytomas. In the murine plasmacytoma system, we have observed that both macrophage and T-cell regulatory cell abnormalities exist. In particular, we found that splenic T cells from mice bearing plasmacytomas can function as very potent inhibitors of antibody synthesis by normal spleen cells in co-culture experiments under certain conditions. However, such suppressor T cells appeared to require a cooperative interaction with normal T cells before a suppressor effect could be observed. The relevant suppressor-type cells express Ly1 and Ly2 differentiation antigens. (We do not yet know whether more than one T-cell subset is involved in the induction of suppression). Because of the results in this section and the previous section, the entire issue of T-T interactions in the generation of regulatory effector cells is becoming a major topic for our research effort.

ALTERATIONS OF IMMUNOREGULATORY CELLS INDUCED BY ANTIBODIES TO Ia ANTIGENS

Background: In mammals, the major histocompatibility complex is a chromosomal segment containing genes that control the strongest allotransplantation antigens. Genes within this region also profoundly influence several functions of the immune system, including the capacity to generate immune responses to certain antigens. The major histocompatibility complex in mice is called H-2 and is located on chromosome 17. At present, five major H-2 regions are recognized: K, I, S, G, and D.

The genetic information for a number of distinct immune functions in mice can be assigned to one or more subregions of the I-region of H-2. Cell surface antigens encoded by genes which map within the I-region are called Ia (immune response associated) antigens. Unlike determinants encoded within the K and D regions of H-2, Ia antigens appear predominantly on B cells and monocytes. However, murine T cells do express some Ia antigens.

The I-region has received enormous attention by investigators throughout the world. By using strains of mice with recombinant chromosomal events within this region, it has been possible to define at least five I-subregions. These are now designated I-A, I-B, I-J, I-E, and I-C. The genetic information for a number of distinct immune functions can can be assigned to one or more specific I-subregions. There is evidence that at least some of the structural information for soluble helper T-cell factors may be encoded in the I-A subregion. Other I-subregions participate in suppressor cell function. For example, some workers have found that suppressor T cells and their factors are capable of inhibiting the mixed lymphocyte reactions of responder cells with the same I-C subregion genotype. Furthermore, the I-J subregion contains the genetic information for surface-membrane markers found on suppressor T cells (and a special subset of helper T cells), and also for determinants found on soluble inhibitory factors derived from such cells. These I-J-related suppressor T-cell factors may profoundly depress both humoral and cellular immune reactions. Under some conditions, such suppressor factors function only in I-J compatible systems and in other circumstances, these factors may function across such histocompatibility barriers.

In humans, the major histocompatibility genes map within the HLA-complex of chromosome 6, and genes closely associated with with HLA-D subregion control the expression of antigens that appear to be the homologues of murine Ia antigens. Sera from multiparous women and allo-sensitized individuals may contain antibodies predominantly reactive to B cells and monocytes, and such alloantibodies have been used to characterize human Ia-like antigens. These antigens exist as a bimolecular glycoprotein complex. They comprise the so-called DR system of HLA-antigens. The DR molecules contain framework antigens (somtimes designated as non-polymorphic components), which can be detected by heteroantiserums raised in rabbits immunized against B-cell membrane glycoproteins with a molecular weight of 28,000-33,000 daltons. Heteroantiserums and alloantiserums directed against human Ia-like antigens appear to interact with the same 28,000-33,000 dalton glycoprotein complex (often abbreviated as p23,30). By analogy to murine systems, human Ia-like antigens are most readily evident on normal (or neoplastic) B cells and monocytes. Human T cells (expecially after exposure to activating stimuli) definitely express Ia-like antigens.

In a number of systems, antibodies directed against Ia antigens have acted as potent inhibitors of immune reactions. Most workers have concluded that such antibodies function by sterically interfering with Bcell or monocyte activity. During the past year, we have studied the mechanisms by which antibodies to human Ia antigens inhibit immune reactions in vitro.

Major Findings:

We observed that both alloantibodies and heteroantibodies directed against human Ia antigens can function as potent inhibitors of humoral immunity in vitro. IgG class antibodies are effective inhibitors at very low concentrations. The inhibitory capacity of such antibodies appears to be critically dependent on the integrity of the Fc portion of the IgG antibody molecule. Using conventional rabbit antibodies, murine monoclonal (hybridoma) antibodies, and human alloantibodies, we found that $F(ab')_2$ fragments depress immune reactions in vitro. This finding suggests that simple steric blockade alone is not the explanation for the inhibitory effect in our hands. (However, steric mechanisms are well documented in other systems).

At relatively low concentrations, we found that the inhibitory activity of antibodies to Ia antigens requires the participation of a radiosensitive subset of normal T cells. We learned that the antibodies to Ia antigens were effective inhibitors when added to lymphocyte cultures either in soluble form, or attached to the surface of macrophages. (However, antibodies directed against other (non-Ia) histocompatibility antigens did not seem to function in this way.) Our data suggest that macrophages with antibodies to Ia antigens attached to their surface membrane can serve as a suppressor signal perhaps through the activation activation of suppressor T cells. Under certain conditions, these suppressor T cells may have their greatest activity against syngeneic B cells. The suppressor T-cell effect brought about by antibodies to Ia antigens may

be mediated by soluble factors under some experimental conditions. These observations might provide new approaches for testing the role of Ia antigens in the cellular reactions that control human immune reactions in normal or pathologic states.

Fc RECEPTORS ON T CELLS

In collaboration with Dr. Werner Pichler, we have extended our studies involving Fc receptors on human T cells. The data thus far suggest that receptors for the Fc-piece of IgM and the Fc piece of IgG cannot be used to classify diffeent subsets of regulatory T cells -- as had originally been accepted by workers in many laboratories. These receptors possibly define different functional stages of normal T-cell activation. Both Fc-IgM and Fc-IgG receptors can be expressed by OKT4 positive T cells (thought to represent a helper/inducer regulatory subset) and also by OKT8 positive T cells (thought to represent a suppressor/cytotoxic subset). However, using a variety of in vitro asays, only Fc-IgM positive T cells are capable of mediating certain T-cell effector functions. For example, only Fc-IgM positive cells can be induced to become effector cells in assays of specific cytotoxic cell mediated lympholysis (CML) reactions, and only Fc-IgM positive cells could be reliably induced to become suppressor cells under conditions of Concanavalin A activation. Moreover, Fc-receptors are not really stable markers because cells may undergo a transition in the expression of relevant receptors. For example, T cells lose Fc-IgG receptors and acquire Fc-IgM receptors after exposure to immune complexes in vitro (and a reverse transition can occur). This process appears to require an intact system of microfilaments and microtubules because the transition from Fc-IgG expression to Fc-IgM expression can be blocked by cytochalasin B and colchicine.

SIGNIFICANCE TO BIOMEDICAL RESEARCH

The recognition that some T-cell leukemias (or lymphomas) may represent a homogenous proliferation of T cells which are programmed for either helper or suppressor function is important for a number of reasons. First, certain forms of humoral immune deficiency may be due to a helper cell deficit, and not totally due to an intrinsic defect in B cells alone or excessive number of suppressor cells. It is possible that certain classes of neoplastic cells or soluble products produced by such cells could prove useful in the treatment of immune deficient states due to thymic dysfunction. Similarly, if such helper cell factors could be isolated (or synthesized), they might be useful in those situations where it is desirable to amplify a normal immune response, for example, following immunizations or in overwhelming infections. In mice, it has been possible to show that there exists a genetically controlled Ly-system of membrane markers which may correlate with helper or suppressor function. Three groups of T cells have been identified in mice. One population bears all three of the so-called Ly determinants ($Ly\text{-}1^+$, 2^+, 3^+). Another population bears only Ly-1 determinants and a third population bears Ly-2,3 determinants. It has been demonstrated that those cells which are Ly 1^+ act as helper cells under some conditions. In addition,

as already dicussed, in mice there is a subregion of the I portion of the major histocompatability complex (designated I-J) which may encode suppressor cell function and certain suppressor cell factors. Furthermore, antisera raised against I-J encoded products may potentiate certain responses. Such anti-I-J antisera may apparently promote tumor rejection in certain systems presumably by eliminating suppressor cells which interfere with host immune responses directed against lethal tumors. The use of neoplastic T cells as starting reagents for the development of appropriate antisera or the isolation of various factors may make it easier to establish and verify serological marker systems for human helper and suppressor function, and may make it possible to serologically manipulate immune responses in certain cancer patients to potentiate tumor rejection or to restore various other elements of the immune system (vide infra). Our preliminary finding that antibodies raised in rabbits immunized with human suppressor-like leukemic T-cells can react with a previously undescribed antigen or set of antigens expressed on cultured T-cells with suppressor function (from unrelated individuals) might be viewed as a positive first step in this overall approach. The use of such neoplastic T cells may also provide a convenient probe to analyze human B cell subsets and to elucidate human T-T interactions more fully.

Turning to our studies relating to multiple myeloma, infections are a very important cause of morbidity and mortality in patients with multiple myeloma. Moreover, there are strong data in a number of systems that neoplastic B cells and plasma cells may still be susceptible to certain physiologic regulatory-cell control mechanisms. Our results suggesting that abnormal suppressor macrophage and T-cell activity may cause or perpetuate the immunodeficiency state associated with this disease might eventually form the basis for new therapeutic strategies. Some of these potential strategies have already been discussed above.

Turning to our studies involving antibodies to human Ia antigens, the knowledge that such antibodies might perturb the immune system in a way which favors the activation of suppressor T cells might broaden the interpretation of a large number of published experiments. Antibodies to Ia antigens may be elicited in a number of clinical circumstances (such as white cell transfusion or organ transplantation). Perhaps the clinical observation that repetitive prior blood transfusions (unmatched for leukocyte antigens) may enhance the acceptance rate in recipients of cadaveric renal transplants could be explained by the generation of selected types of antibodies to Ia antigens with secondary (protective) suppressor cell formation. Recent observations suggest that Ia-like molecules may be expressed on neoplastic cells that do not have a lymphocyte origin (e.g., malignant melanoma cells). These tumor associated Ia-like molecules may have significant structural differences from normal autologous Ia molecules. Thus, it is conceivable that certain anti-tumor antibodies are directed against Ia-like antigens. It is also conceivable that in some circumstances such antibodies could play a role in the generation of suppressor T cells that are known to be associated with certain tumors.

Proposed Course:

During the coming year we will continue our characterization of the immunoregulatory properties of lymphocytes with T-cell leukemias and lymphomas. One of the highest priorities will be to learn whether it is possible to generate stable and functional T-T hybridomas by fusing defined regulatory neoplastic cells with appropriate cell lines. We will explore whether inducing factors can bring about the maturation of other T-cell neoplasms.

We will continue our studies involving the regulatory cell abnormalities associated with plasma cell neoplasms We will attempt to define whether the suppressor T cells seen in mice with plasma cell tumors bear characteristic surface membrane markers. We will also attempt to define the precise inter-relationship between the suppressor macrophages and suppressor T cells observed in these systems.

We will make an attempt to elucidate how antibodies directed against Ia antigens inhibit immunity. We will test other kinds of antibodies to human Ia antigens (including monoclonal antibodies produced by somatic-cell hybridization and cloning technology). We will attempt to define what genetic barriers (if any) may restrict the suppressor T-cell activity induced by antibodies to Ia antigens either in soluble form or attached to macrophage surface membranes. We will also attempt to identify the soluble factor(s) that may mediate the suppressor T-cell effect.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-04015-11-MET

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Development and Function of Humoral and Cellular Immune Mechanisms of Host Defense

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Jane Grayson, M.D.,	Expert	MET	NCI
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6	4	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study is directed towards determining mechanisms important to the development and function of humoral and cellular immune responses; the nature of deficiency in immune function in patients with primary immunodeficiency or immunodeficiency secondary to malignancy; and developing approaches to prophylaxis and/or therapy of infections and neoplastic disease associated with immune processes.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The objectives of this study were to determine mechanisms important to the development and function of both humoral and cellular immune responses in man and animals; to determine the nature of the deficiency in immune function characterizing such disease states as hypogammaglobulinemia, ataxia telangiectasia, the Wiskott-Aldrich syndrome, intestinal lymphangiectasia, and advanced malignancy; and to develop approaches to prophylaxis and/or therapy of the infections and neoplastic disease frequently associated with defective immune processes. This plan is directly related to Objective 2, Approach 1 of the National Cancer Plan.

Methods Employed: Antibody responses of experimental animals and man were determined after immunization with a variety of antigens and the antibodies were detected by standard techniques or methods developed in our laboratory. Cellular immune responses were measured by delayed hypersensitivity skin testing, testing for contact sensitivity to dinitrochlorobenzene and by skin allograft rejection. Proliferative responses in vitro were tested using nonspecific mitogens, specific antigens, and allogeneic cells in mixed lymphocyte culture. Immunoglobulin secretion by activated lymphocytes was measured by a newly developed reversed hemolytic plaque assay and this test was applied to cultured cells stimulated with a variety of mitogens, viruses, and chemical agents. Studies of the characteristics of various cell surface receptors on immunocompetent lymphocytes and monocytes-macrophages utilized autoradiography, immunofluorescence microscopy, and cellular rosette formation.

Major Findings: The interactions between immunocompetent cells and the mechanisms of immunoregulation have been a major interest of the Cellular Immunology Section for several years and a variety of techniques and model systems have been developed for these studies. Recently we have developed an entirely new approach to the investigation of B cell differentiation which permits the precise quantitation of the numbers of lymphoid cells secreting each class of immunoglobulin. This technique called the reverse hemolytic plaque assay has been employed in both in vivo and in vitro studies of normals and patients with a variety of immunodeficiency, autoimmune, and malignant disorders.

We have previously reported studies in which the reverse hemolytic plaque assay was used to study the activation and differentiation of the B lymphocyte series in vitro. The reverse plaque assay differs from other techniques used in the Branch for the study of B cell differentiation in that it detects individual cells actively secreting immunoglobulin at any given point in time. Thus, this method provides an important complementary approach to the radioimmunoassay which is used to measure the cumulative immunoglobulin molecules secreted into the culture supernatents. Kinetic studies of the presence of immunoglobulin secreting cells in cultures of blood lymphocytes stimulated with the polyclonal mitogen, pokeweed mitogen, showed that on day 3 of culture an exponential rise in the number of immunoglobulin secreting cells began and the response peaked at 4-6 days of culture. This pokeweed mitogen stimulated response

was shown to be dependent upon the interaction of T cells, B cells and macrophages in these cultures.

The Epstein-Barr virus (EBV) is the etiologic agent of acute infectious mononucleosis in man and has been closely associated with African Burkitts lymphoma, nasopharyngeal carcinoma and rare cases of agammaglobulinemia. The virus is unique among human pathogens in that is specifically infects cells of the immune system, the B lymphocytes, and transforms these B cells into permanently growing cell lines in culture. We studied the effect of EBV on human peripheral blood lymphocytes in culture utilizing the reverse hemolytic plaque assay. EBV added to lymphocytes in culture induced the B cells to differentiate into cells actively secreting each class of immunoglobulin. Cell separation experiments demonstrated that EBV induced activation of B cells was totally independent of T lymphocytes in contrast to pokeweed mitogen which is dependent on T cells for its activation of B cells. T lymphocytes, however, may exert a profound regulatory influence on EBV induced B cell activation, even if they are not required for EBV to induce B cells to differentiate into immunoglobulin producing cells. This T cell regulatory effect is seen most strikingly in patients with acute infectious mononucleosis. During the first week of infection, markedly elevated numbers of immunoglobulin secreting cells were found in the blood of these patients associated with acutely elevated serum immunoglobulin levels. These increased levels of immunoglobulin secreting cells and serum immunoglobulins probably reflect the in vivo activation of B cells by the Epstein-Barr virus. After about one week of this illness, the lymphocytes from patients with infectious mononucleosis were found to be unresponsive in vitro in that they would no longer differentiate into immunoglobulin secreting cells in response to either pokeweed mitogen or exogenous Epstein-Barr virus. Co-culture experiments showed that this lack of response was due to intense suppressor T cell activity which had developed in these patients. Following the appearance of these suppressor T cells the number of immunoglobulin secreting cells in the blood of infectious mononucleosis patients fell to very low levels and globulin secreting cells in the blood of infectious mononucleosis patients fell to very low levels and returned to normal only after the suppressor T cell activity had disappeared associated with clinical recovery. Thus in this disease as opposed to the situation seen in agammaglobulinemia where suppressor T cells may lead to the clinical disorder, suppressor T cells appear to serve a unique role in host defense by inhibiting the proliferation and activation of the target cell of the virus and thereby limiting virus propagation. However, we have also studied one patient with agammaglobulinemia which developed following a documented case of infectious mononucleosis caused by Epstein-Barr virus. In this child, very intense suppressor T cell activity was demonstrated suggesting that the physiologic suppressor response to the Epstein-Barr virus infection in this child was itself not normally regulated, and thus its persistence caused permanent agammaglobulinemia.

A genetic restriction of the interaction between cells of the immune system has been repeatedly demonstrated in certain systems, while cellular cooperation in other immune systems occurs readily across allogeneic barriers. With pokeweed mitogen as a stimulant of B cell differentiation in culture, T cells, B cells, and macrophages from individuals differing in genetic makeup can cooperate with each other to produce a normal in vitro response. Thus T cells obtained from one subject can help B cells from another subject to produce immunoglobulin in vitro. Even though such a mixture of allogeneic cells may result in a mixed lymphocyte reaction occuring in such cultures, such a mixed lymphocyte reaction appears to have no significant consistent effect on the ultimate response of the B cells in such culture. Although Epstein-Barr virus does not require T cells for its ability to activate B cells to produce immunoglobulin, we have observed that the presence of allogeneic T cells in such cultures has a profound effect on B cell differentiation when normal T cells from one individual are mixed with B cells from another and the co-culture is stimulated with Epstein-Barr virus. In a majority of cases such cultures fail to produce immunoglobulin. If the allogeneic T cells have been irradiated with 2000 R, the B cells are capable of responding and producing a normal immunoglobulin response suggesting that in the allogeneic co-culture an active process of suppression is occuring which prevents the EBV induction of B cell activation while at the same time having no significant effect on the capacity of pokeweed mitogen to induce B cell activation. In order to determine whether this suppressor effect observed in allogeneic cultures stimulated with EBV was related to antigens encoded by the major histocompatibility complex in man, family studies were undertaken. Such studies demonstrated that when family members shared all antigens of the major histocompatibility complex no suppressor effect was observed in co-culture. When members of families differed at antigens in the major histocompatibility complex then the suppressor effect was observed, demonstrating that this genetic restriction was linked to HLA, the major histocompatibility complex of man. Further studies of co-cultures between B cells and T cells of individuals differing by single or multiple antigens in the HLA complex were then undertaken. It was shown that the suppressor reaction that occured in such allogeneic mixed cultures did not occur when the only differences in such culture were at the HLA-D locus. Thus, even though a significant mixed lymphocyte reaction might be occuring in culture no suppression was found when the only genetic difference was at the D locus. By contrast in allogeneic cell mixes in which D locus identity was present, but in which significant differences occurred at the A or B locus antigens, marked suppression of the EBV response was seen. Thus the activation of suppressor T cells effective in inhibiting EBV induced B cell activation by allogeneic interactions is tightly linked to recognition of A and B antigens of the HLA major histocompatibility complex in man. Studies are now in progress to use this observation of allogeneic suppression as a tool to study suppressor mechanisms in patients who may have disorders of immunoregulation.

The persistance of the EBV genome in B lymphocytes from persons long since recovered from infectious mononucleosis has been repeatedly demonstrated. Thus lymphocytes from such individuals, when placed in tissue culture will spontaneously give rise to continuous B cell lines containing EBV. The mechanism which prevents this B cell transformation from occuring in vivo has been difficult to define. The highly activated suppressor T cells which can be demonstrated in infectious mononucleosis are not demonstrable in recovered patients. However, we have found that normal EBV sero positive donors have another type of suppressor T cell mechanism which becomes activated in cultures of their lymphocytes exposed to EBV. This suppressor T cell exerts its regulatory effect after 7-10 days of culture and then totally shuts off further virus induced B cell activation. EBV sero negative individuals do not have such a late acting suppressor T cell population. Studies of this form of immunoregulatory T cell in patients who develop EBV associated malignancy such as Burkitt's lymphoma would be particularly interesting and we hope to pursue such studies in the near future.

Several lines of evidence suggest a potential relationship between EBV and the disease, rheumatoid arthritis. Patients with RA have elevated serum antibody titers to certain EBV associated antigens, EBV rapidly induces B cell lines from RA patients' peripheral lymphocytes, and EBV infected lymphocytes in culture will produce rheumatoid factor. We have studied immunoregulatory T cell function in 20 patients with RA to determine whether a defect in such function may contribute to the immune disorder underlying this disease. Helper T cell activity for pokeweed mitogen induced immunoglobulin production was normal in these patients as was the suppressor T cell function activated in response to allogeneic histocompatibility antigens. Strikingly, however, the late acting suppressor T cell which inhibits EBV induced B cell activation in normal immune subjects was markedly deficient in these patients even though they were immune to EBV as determined by serum antibodies to the virus. Thus, patients with this disease exhibit a profound defect in immunoregulatory T cell function which is restricted to involve the response to only a specific agent, the Epstein-Barr virus. Since EBV persists indefinitely in the genome of B lymphocytes after primary infection and since this virus can activate B cells to produce immunoglobulin, it represents a potential perpetual stimulus for production of antibody and rheumatoid factor. Thus, the defect in specific immunoregulatory T cells which prevent EBV induced B cell activation may be an important contributor to the immunologic basis of this disease.

The Epstein-Barr virus has been widely studied in laboratories all over the world and a number of different strains of this virus have become available. All of our previous studies have utilized the B 95-8 strain of EBV, orginally isolated from a patient with acute infectious mononucleosis. As we have mentioned, B 95-8 strain is independent of the effects of T cells and monocytes for its capacity to activate B lymphocytes to produce immunoglobulin, and this strain also transforms B lymphocytes into permanently growing cell lines. The P3HR-1 strain of EBV was originally isolated from a patient with Burkitts lymphoma. This strain differs from

B 95-8 in that it is not a transforming virus. That is, this virus does not induce B cells to develop into permanently growing cell lines. In studies with P3HR-1 virus in culture it was found that this virus also induced the activation of B lymphocytes into immunoglobulin secreting cells. This demonstrates that the capacity of the virus to induce transformation in the B cells is not a requisite for that virus to activate B cells to become immunoglobulin secreting cells. Interestingly when T cells and B cells were separated into individual populations and then stimulated with P 3 HR-1 EBV, the B cell population alone would not respond with the production of immunoglobulin secreting cells. Only when T cells were present would this strain of Epstein-Barr virus activate B lymphocytes to become immunoglobulin producing cells. A third strain of Epstein-Barr virus named Ag 876 was also found to require the presence of normal T cells in order for it to activate B lymphocytes to produce immunoglobulin. Interestingly, this virus strain would induce the transformation of B lymphocytes into permanently growing cell lines. Thus, the capacity of the Epstein-Barr virus to activate B lymphocytes is independent of the capacity of this virus strain to transform B cells into permanent lines and the activation of immunoglobulin secreting cells by the virus may in certain circumstances require the presence of T lymphocytes. We hope to exploit the differences observed in these Epstein-Barr virus strains to help us explore in greater detail the interactions between virus and immunocompetent cells and the effects that mutations in the virus may have on its ultimate biologic activity.

The Epstein-Barr virus has also proved to be a useful in vitro probe for the study of the development of immune competence in human cells. The human newborn has been repeatedly demonstrated to have deficient immune responses. We studied the responses of human cord lymphocytes in vitro to stimulation with the polyclonal mitogens, pokeweed mitogen, and with Epstein-Barr virus. Cord blood lymphocytes produced no immunoglobulin in response to pokeweed mitogen, in marked contrast to the effects of this mitogen on adult peripheral blood cells. However, cord blood lymphocytes were capable of producing immunoglobulin when stimulated with Epstein-Barr virus. Co-culture experiments with mixes of cord blood and adult lymphocytes also failed to respond to pokeweed mitogen demonstrating that the cord blood cells have a suppressor T cell population which prevents the differentiation of B cells. This suppressor T cell present in cord blood is unlike that seen in patients with agammaglobulinemia or infectious mononucleosis in that it does not inhibit Epstein-Barr virus induced B cell activation. This apparent paradox in the capacity of the suppressor cell to inhibit thymic dependent responses while having no suppressor activity on thymic independent B cell activation was resolved by the demonstration that double mitogen experiments, that is, culture stimulated with both pokeweed mitogen and Epstein-Barr virus, also had deficient B cell responses due to suppression. Thus, cord blood contains a population of T cells that are activated to become suppressor cells in the presence of pokeweed mitogen, a cell population quite different from that found in adult blood.

Extending our studies on the development of immunocompetence in neonatal lymphocytes, we evaluated T helper cell activity by comparing the capacity of irradiated cord blood or adult T cells to help cord blood or adult B cells produce immunoglobulin in response to pokeweed mitogen. We found that cord blood T cells possessed helper activity for IgG, IgA, and IgM when tested with adult B cells, and that this helper activity was similar in magnitude to adult T cells for IgM and IgG, but slightly impaired for IgA. Interestingly, when tested on cord blood B cells, cord blood T cells had significantly lower helper activity than did adult T cells. In further examining the responses of neonatal B cells , we found that only IgM was produced in response to either EBV or PWM, rregardless of whether adult or neonatal T cells were used to supply helper activity. Another interesting difference between adult and cord blood B cells was found in their suceptibility to suppression by alloantigen activated T cells. As described above, alloantigen stimulated T cells suppress the response of adult B cells to EBV. However, the neonatal B cell response to EBV was unaffected by the presence of allogeneic T cells. Thus, the immunocompetent cells of the human newborn differ from adult cells in several important aspects. Neonatal B cells are restricted in their capacity to produce immunoglobulin in that only IgM, but IgG or IgA is produced. Furthermore, neonatal B cells are insensitive to suppressor influences of alloantigen activated adult T cells and they are less responsive than adult B cells to the helper activity provided by neonatal T cells. In addition, the human newborn possesses a population of suppressor T cell precursors which are more readily activated for suppressor function by PWM than are adult T cells.

The mechanisms and sites of action of suppressor T cells have been a major area of interest in many laboratories. In murine systems, many suppressor T cells which inhibit B cell function appear to do so by neutralizing or inactivating helper T cells required for the particular B cell response being measured. In most of the suppressor cell assays useful for studies of human B cell responses, the site of action of the suppressor cell is unknown. The Epstein-Barr virus offers a unique tool to address this issue since it is capable of activating B cells in the complete absence of helper T cell influences. Thus, if suppression of EBV induced immunoglobulin production is observed, such suppression must be acting directly on the B cell. From the studies described in the preceeding paragraphs we have been able to define four separate and distinct suppressor T cell systems, and all four of these suppressor T cells act directly on the B cell since they suppress the EBV induced response of these cells. In infectious mononucleosis, suppressor T cells are present which inhibit all B cell activators, both helper T cell dependent ones such as PWM, and helper cell independent activators such as EBV. In the human neonate, such polyclonal suppressor cells are not found circulating in an activated state, but after stimulation with PWM they become activated and also then inhibit EBV induced responses. In EBV immune subjects, we observed a unique late acting suppression which was specific for EBV alone, not inhibiting PWM induced responses. This suppressor T cell then also acts only on B cells. The fourth suppressor system was that observed in cultures containing mixtures of cells from individuals who differed significantly in their major histocompatibility

antigens. In this circumstance suppressor T cells become activated which inhibit the EBV, but not the PWM, induced B cell response. Again, this suppressor mechanism must act directly on the B cell, but in this case, only that subpopulation of B cells which responds to EBV.

In studies of the suppressor T cells present in certain patients with hypogammaglobulinemia, it has been observed that these suppressor cells are sensitive to both irradiation and to hydrocortisone. Thus steroids or irradiation are able to block the suppressor activity of such cells. During the course of these experiments we observed that hydrocortisone alone cultured with normal lymphocytes seemed to induce immunoglobulin production in culture. Human peripheral blood lymphocytes cultured with hydrocortisone or many of its analoges including dexamethasone, prednisolone, and methyprednisolone were found to produce in culture as many immunoglobulin secreting cells as did cultures stimulated with pokeweed mitogen. Kinetic studies showed that this response to steroid hormone in vitro began to appear at a about the 4th day and peaked somewhat later than pokeweed mitogen at the 8th to the 10th day of culture. Other steroid hormones such as dyethylstilbesterol and testosterone did not induce the activation of B cells in culture. Aldosterone, a steroid hormone with weak glucocorticoid activity, stimulated B cells to produce immunoglobulin only when used at very high concentrations in vitro. In order to determine the cellular basis for such stimulation, peripheral blood lymphocyte populations were separated into T cells, B cells and monocytes. The glucocorticoids were found to be unable to stimulate immunoglobulin production by purified B lymphocytes alone. Both monocytes and T lymphocytes were required in the cultures for glucocorticoids to have their stimulatory activity on B lymphocytes. Since glucocorticoids are able to reverse the suppressor T cell activity seen in certain pathologic conditions and also appear to be able to reverse normal suppressor T cell activity, a potetential mechanism for the action of steroid hormones in inducing B cell activation would be the inactivation of a suppressor cell maintaining such B lymphocytes in an inactive state. T cells irradiated with 2000 R to inactivate the radiosensitive suppressor T cell were mixed with B lymphocytes in culture. Such cell mixtures produced no more immunoglobulin than that produced by B lymphocytes alone. However, when corticosteroids were added to such mixtures a marked increase in immunoglobulin production was observed. Thus although we have not been able to directly demonstrate that corticosteroids are not simply inactivating a suppressor T lymphocytes, other procedures which inactivate supressor T cells do not result in the activation of immunoglobulin production by B cells without the addition of the glucocorticoid hormones.

To further examine the cellular sites of action of the steroid effect, soluble factors produced by cultured T cells were tested for their ability to substitute for intact T cells in this response. Supernatants from T cells cultured without stimulants for 24-48 hours were added to B cell cultures. Such supernatants were incapable of substituting for intact T cells when the cultures were stimulated with pokeweed mitogen, but they were able to replace T cells when corticosteroids were used as the stimulant. Thus, it appears that the principal site of action of steroid

hormones in this system is upon the B lymphocytes. However, the B cell response to steroids is also clearly restricted to only a subpopulation of B cells. For example, human neonatal B cells do not respond with immunoglobulin production following corticosteroid stimulation. In addition, IgE production is only minimally enhanced in normal subjects with steroid stimulation while large amounts of IgE are produced by by lymphocytes from allergic subjects when stimulated with steroids. This lack of stimulation of cord blood lymphocytes, the preferential stimulation of IgE production in allergic subjects, and the lack of detectable proliferation in steroid stimulated cultures actively producing immunoglobulin, have led us to propose that steroids are inducing a subset of memory B cells to produce immunglobulin in vitro.

The Wiskott-Aldrich syndrome is a primary immunodeficiency disease characterized by thrombocytopenia with frequent hemorrhage, excema of the skin, and recurrent infections with all classes of micro-organisms. We have had a long term interest in this disorder and have defined a great many defects in the immune system of such patients. Splenectomy as a therapy for the thrombocytopenia in this disease has been considered to be contraindicated because many patients treated with this procedure have died within a few weeks of surgery because of overwhelming infection. We have recently completed a study of the effects of splenectomy on the thrombocytopenia in this disease and the mechanisms involved in the response to such treatment. Of the 47 cases of the Wiskott-Aldrich syndrome seen at the NIH, 17 have been treated with splenectomy. All 17 patients had a normalization of their platelet count following removal of the spleen. In studies of the survival of the patients following splenectomy, it was found that patients maintained on prophylactic antibiotics following the procedure had done exceptionally well with an average survival of greater than 11 years post splenectomy. By contrast, patients who were not treated with prophylactic antibiotics died of infection within a few months of surgery. Thus splenectomy combined with the use of prophylactic antibiotics seems to provide a useful therapeutic approach to the problem of recurrent hemorrhage in patients with this disease. One of the unique features of the thrombocytopenia in the Wiskott-Aldrich syndrome is that the platelets in the circulation of these patients are very small in size. This micro-thrombocytopenia has not been described in other forms of thrombocytopenia and thus appears to be unique to this syndrome. Interestingly, following splenectomy of these patients when their platelet count returns to normal, the platelet size also returns to the normal range. Thus, the platelet abnormality in the Wiskott-Aldrich syndrome appears to reflect an extrinsic process of destruction mediated by the spleen rather than reflecting an intrinsic abnormality in the production or survival of platelets. Most recently in an attempt to define potential mechanisms for such a destructive process, we have been investigating the possibility that the platelets from children with this disease might be effected by a form of autoimmune process. We have found in all 15 patients thus far studied a marked increase in platelet bound antibody. Following splenectomy, the level of platelet bound IgG falls rapidly as the platelet count returns to normal. If a relapse in the thrombocytopenia occurs, the platelets once again become heavily coated with anti-platelet antibody.

Significance to Biomedical Research:

The present studies extend our understanding of the diverse, yet interrelated mechanisms contributing to the development and normal functioning of immune system in animals and man. They indicate that the normal expression of immune function is dependent on multiple processes and that defects in immunity may be the result of factors influencing a variety of these processes. For example, disfunction of the immune system can occur through such diverse factors as defects in differentiation on the one hand or through the mediation of exogenous viral pathogens on the other. Thus human disease may result from a deficiency of immune elements as in certain forms of agammaglobulinemia or from an excess of certain immune functions as in other types of agammaglobulinemia associated with excessive suppressive T cell activity. Suppressor T cell function may be part of a normal defense mechanism in such diseases as infectious mononucleosis and a deficiency of normal suppressor T cell activity may lead to the expression of certain autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus. In other disorders such as sarcoidosis and idiopathic pulmonary fibrosis, the recognition of immune elements participating in the pathogenesis of disease is just appearing and hopefully these insights will provide for new approaches to the understanding and therapy of these disorders.

Proposed Courses:

Continue the studies directed toward the understanding of the pathways involved in the development and function of normal immune responses and the application of insights gained through such studies for the development of new approaches for the prevention and therapy of human disease.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-04018-05-MET

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Study of Human Immune Defense Mechanisms and Its Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Andrew V. Muchmore, M.D.	Senior Investigator	MET	NCI
OTHER:	Samuel Broder, M.D.	Clinical Associate	MET	NCI
	R. Michael Blaese, M.D.	Senior Investigator	MET	NCI
	Eugenie Kleinerman, M.D.	Clinical Associate	MET	NCI
	Robert Hall, M.D.	Clinical Associate	MET	NCI
	Dean Mann, M.D.	Senior Invest.	IMM	NCI

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Metabolism Branch, DCBD, NCI

SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are aimed at understanding how the human immune system recognizes and destroys foreign cells. Special emphasis is placed on early antigen nonspecific cytotoxic responses (spontaneous cytotoxicity), which precede specific immune recognition. Both cell mediated and humoral mediated forms of spontaneous cytotoxicity have been examined. A different project has used the in vitro addition of both simple carbohydrates and defined anti DRw specific antisera disect cellular control phenomena. These studies have demonstrated a profound negative regulatory effect by both anti DRw antisera and simple carbohydrates.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The objectives of these studies were to 1) delineate the importance in man of a newly recognized system of non-specific cell mediated spontaneous cytotoxicity, 2) examine the effect of antisera which recognizes gene products encoded by the human Drw locus on assays of cellular and human immunity, 3) characterize a newly recognized class of surface receptors for the Fc portion of human immunoglobulin on human T cells, 4) examine the phylogeny of antigen non-specific cytotoxicity systems in vivo 5) assss the molecular mechanisms involved in some forms of cell-cell cooperation, and 6) asses the effect of various chemotherapeutic anti-neoplastic drugs on spontaneous cytotoxicity.

Methods Employed: In vitro cellular cytotoxicity systems have been developed for antibody dependent cellular cytotoxicity, mitogen induced cellular cytotoxicity, cell mediated lympholysis, and spontaneous monocyte mediated cytotoxicity using a sensitivity micro ^{51}Cr release assay for various target cells. These results were correlated with standard in vitro and in vivo assays of cell mediated and humoral mediated immunity. For the characterization of immunoglobulin class specific Fc receptors on human T cells, purified IgG, IgM, and IgA chromatography, gel filtration and antigen specific absorption of certain monoclonal mouse myeloma proteins.

Major Findings: Our laboratory is continuing four major projects and is embarking on a fifth. Dr. Robert Hall has been working for the last year characterizing the molecular requirements for the expression of synergistic cytotoxicity. This model which measures the ability of human serum factors to activate human monocytes and lyphocytes and to kill erythrocyte targets is now substantially characterized. Homogenous purified complement components obtained in collaboration with Dr. Harvey Colton of Boston have been used and we have shown that C5 and Factor B in the presence of activated human monocytes (which fail to kill by themselves) are necessary and sufficient to induce lysis. Lymphocytes also exhibit killing in this model and require at least C7 and C8 as well as C5 and factor B. This research is important not only because it represents a unique model of cell mediated cytotoxicity but also because it unites antigen idependent fluid phase lysis (alternate complement pathway) with antigen independent cell mediated lysis (NK and spontaneous cytotoxicity).

Dr. Eugenie Kleinerman has continued her work characterizing the pharmacology of spontaneous monocyte cytotoxicity, a model which measures cell mediated lysis of target cells in the absence of serum factors. Previous work from our laboratory has shown that cytotoxic monocytes are under the control of suppressor cells. Dr. Kleinerman has shown that Cis Pt Adriamycin, x-irradiation and 1 phenyl alanine mustard all paradoxically enhance monocyte function. Each drug or treatment exhibits unique time constraints and cellular targets. For example x-irradiation enhances killing by inactivation of suppressor cell activity while Cis Pt directly activates monocyte function. These studies have been expanded to clinical studies of cancer patients and early results demonstrate excellent

correlation with in vivo and in vitro data. These studies are extremely important because they imply a unique mechanism resulting in actual activation of cytotoxic monocytes by agents which are normally thought of as toxic. Such activation may play an important in vivo role. We have also studied anti-inflammatory drugs used in the treatment of rheumatoid arthritis (ASA, Indocin, steroids and gold) and find that these two can enhance monocyte function.

We have continued a very basic thrust of our laboratory attempting to characterize sugar specific cell mediated recognition. We believe that certain sugars, L-rhamnose, D-mannose, and N acetyl glucosamine act by preventing different forms of cellular recognition phenomena. We have attempted to further characterize the mannose receptor in collaboration with Dr. Alan Rosenthal of Merck.

Finally, we have begun to re-examine the phenomena of suppression of in vitro T cell reactivity by compounds found in human pregnancy urine. Previous work from our lab has shown that HCG is not responsible which has been continued by several workers. We are now purifying small molecule weight glycoproteins from pregnancy urine using lectin columns. Even though the study is in it's infancy, we have isolated compounds which suppress at nanograms/ml (approximately 1000 times more potent than HCG.

Significance to Biomedical Research: Our studies on both monocyte mediated cellular cytotoxicity and factor B dependent cell mediated cytotoxicity have several major implications. Our data suggest that a variety of "nonspecific" in vitro and killing assays are in reality quite specific and represent target spcific cell surface sugar recognition. Our phylogenetic studies imply this phenomonen is ubiquitous. We believe this type of cytotoxicity represents an important aspect of host immune defense which is independent of prior antigen exposure. From a clinical perspective, we have evidence that both therapeutic levels of x-ray and the radiometric drug Cis PPD enhance in vitro killing. This enhancement occurs via different mechanisms ... x-ray inactivates a suppressor cell while Cis PDD appears to directly activate monocytes. We propose that this phenomena may be an important mechanism by which these agents exert their anti-neoplastic effects in vivo as well as their well described direct toxicity towards tumor cells. We have recently analyzed a large group of patients with malignancy and have shown them to have defective in vitro monocyte mediated killing. Our evidence suggests that successful Cis PDD treatment results in restoration of monocyte cytotoxicity in vitro and in vivo. Our data concerning the effect of various sugars on in vitro assays of antigen specific proliferation have enormous implications concerning how cells communicate with each other. At this juncture we are postulating that carbohydrate recognition plays a central role in self-self and self-non-self recognition phenomena.

Proposed Course: Our laboratory will continue to characterize the nature of carbohydrate rceptors and the effect of blocking these receptors on in vitro and in vivo immune reactivity. We will extend our studies to an in vivo model in efforts to control immunologic responses in an intact

animal in order to gain insight into possible approaches and therapy of diseases which are the result of over active immune responses. We are embarking on a major effort to screen chemotherapeutic anti neoplastic agents both in vivo and in vitro to asses their capacity to activate monocyte mediated killing.

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Lawrence, E.C., Arnaud-Battandier, F., Grayson, J.G., Koski, I.R., Dooley, N.J., Muchmore, A.V., and Blaese, R.M.: Ontogeny of humoral immune function in normal chickens: a comparison of immunoglobulin secreting cells in bone marrow, spleen, lungs and intestine. Clin. Exp. Immunol. 43: 450-457, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04017-05-MET

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biology of the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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SECTION

Immunophysiology

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were undertaken to investigate specific immunologic responses by human peripheral blood leukocytes from normal individuals and patients with immunologic deficiency states. Methods were developed to measure specific cytotoxic T-cell responses and humoral antibody responses by B-cells in vitro. Patients with immunodeficiency diseases were heterogeneous with regard to their ability to generate cytotoxic T-cells in vitro. Most patients with hypogammaglobulinemia produced immune cytotoxic T-cells normally, while patients with the Wiskott-Aldrich syndrome and ataxia telangiectasia produced almost no immune cytotoxic T-cells. Defects in the production of immune cytotoxic T-cells may contribute to the increased incidence of neoplasia observed in immunodeficiency diseases.

Project Description:

Objectives: Exposure to infectious agents and other foreign antigens elicits biologic response phenomena in host tissues which may confer protection to the host organism upon subsequent exposure to the same antigen(s). These biologic phenomena are termed immune responses and have generally been divided into two major types 1) cell-mediated responses which require the immediate presence of immune cells for their effects to be manifest, and 2) humoral responses in which cells elaborate soluble molecules such as antibody which may exert their protective effects at some distance from the immune cells which produce them. Studies were undertaken to investigate the biology of cellular and humoral immune responsiveness in normal individuals and in patients with a variety of immunodeficiency diseases in which congenital and acquired defects in host responsiveness are associated with an increased incidence of infection and malignant tumors. Particular emphasis was placed on studying the mechanisms underlying perturbations of immune function in these patients which might account for their increased incidence of neoplasia.

Methods Employed: Over the past year, special emphasis was placed on the study of antigen-specific cellular and humoral immune responses by human peripheral blood leukocytes in vitro. Two major classes of immune responses have been under investigation: 1) cell-mediated cytotoxic responses by leukocytes whose development is thymus dependent (T-cells), and 2) humoral antibody responses produced by a second class of leukocytes, (B-cells) whose development is bone marrow dependent and requires T-cells for full maturation. Both T-cell and B-cell immune responses are highly dependent on monocytes which are a third type of mononuclear leukocyte. In vivo, these cellular and humoral responses normally occur concomitantly in a highly ordered fashion with multiple cellular interactions occurring among sub-populations of T-cells, subpopulations of T-cells with B-cells and macrophages, and B-cells with macrophages. These cellular interactions result in a finely regulated response which is promptly initiated and appropriately terminated following antigenic exposure.

A major component of host responsiveness to a variety of antigens may take the form of tissue destruction (cytolysis) whereby antigen bearing cells are lysed and eliminated. Cells capable of directly mediating such cytolytic events are termed cytotoxic effector cells. Although we have demonstrated that several types of leukocytes can function as cytotoxic killer cells, we have recently focused our attention on cytotoxic T-cells which possess specific cell surface receptors for antigens expressed on those cells which undergo lytic events (target cells). Cytotoxic immune T-cells are undetectable in the peripheral blood of non-immunized individuals. Human cytotoxic T-lymphocytes (CTL) with receptor specificities for surface antigens present on the cells of unrelated individuals may be generated during seven days of in vitro culture in which density gradient centrifugation prepared responder peripheral blood mononuclear leukocytes are immunized by co-culture with monoclear leukocytes from a non-related individual whose proliferative capacity has been inhibited by irradiation. In such cultures, responder CTL

precursors proliferate and mature into cytotoxic effectors whose lytic activity is measured by a 6 hour radioisotopic release assay employing ^{51}Cr labelled target cells from the stimulating cell donor. These immune CTL recognize a series of target cell surface molecules termed transplantation or histocompatibility antigens, which differ among individuals of the same species (alloantigens) and these cytotoxic effectors are therefore termed alloimmune CTL. Alloimmune CTL probably play a major role in the host rejection of histoincompatible allografts such as that occurring in kidney or heart transplantation and also in graft versus host disease in which an immunoincompetent host receives a graft of immunocompetent T-cells and the donor graft attacks host tissues.

Immune CTL have also been produced with receptor specificities for various chemical and viral antigens. Studies have demonstrated that individuals immunized either in vivo or in vitro with chemical haptens or viral antigens produce CTL which will lyse homologous antigen bearing autologous (self) target cells but will lyse antigen bearing target cells from other individuals only when the immune CTL and the target cell share genetically determined histocompatibility antigens. The relevant histocompatibility antigens which must be shared between targets and immune CTL are those encoded by a genetic locus termed the major histocompatibility complex (MHC) which in humans codes for the human leukocyte antigens (HLA) A, B, C, DR, and D.

These cytotoxic T-cells with receptor specificities for foreign antigen plus autologous MHC antigens are termed self-MHC restricted CTL. Evidence from studies in experimental animals has suggested that self-MHC restricted CTL play a major in vivo role in the recovery from virus infections, immunologic surveillance against neoplasia, and deleterious autoimmune phenomena. In humans we have produced CTL with receptor specificities for chemical haptens (trinitrophenol-TNP) and autologous MHC antigens in vitro by seven days primary immunization of responder peripheral blood mononuclear leukocytes with hapten modified autologous leukocytes, followed by five additional days of secondary in vitro stimulation with fresh or cryopreserved autologous haptenated leukocytes. Lytic T-cell activity is detected by a 6 hour radioisotope release assay employing ^{51}Cr labelled TNP-modified target cells. Human CTL with specificities for viral antigens and self MHC gene products are produced in vitro by culturing responder peripheral blood mononuclear leukocytes with infectious allantoic fluid containing influenza A/Hong Kong virus or irradiated influenza virus infected autologous leukocytes for seven days. Such cultures proliferate and generate CTl whose lytic activity is tested on autologous or allogeneic virus infected targets in a short term ^{51}Cr release assay.

The second major area of emphasis centered on the assessment of humoral immune function by human peripheral leukocytes in vitro. In these studies we concentrated on measuring specific humoral antibody production by B-lymphocytes. The presence of specific antibody in biological fluids can be detected by a variety of methods including agglutination with particulate antigens, hemolysis employing antigen coated erythrocytes and radioimmunoassays. Each of these methods offers differing levels of sensitivity in detecting specific antibody. Recently, a very sensitive technique for measuring specific antibodies in the

nanogram to picogram per milliter range has been described which involves the detection of antigen bound immunoglobulin thru an enzyme-conjugated second antibody directed against the bound first antibody. This technique has been termed the Enzyme Linked ImmunoSorbent Assay (ELISA). In a usual assay, antigen (i.e. virus) is first allowed to bind nonspecifically in the wells of a multiwell plastic plate and non-bound antigen is washed away. A source of putative antibody (human serum or in vitro human leukocyte culture supernatant) against the bound antigen is then added, incubated, and non-bound antibody is washed away. Next, an enzyme (alkaline phosphatase)conjugated heterologous (rabbit) antibody directed against human antibody is added, incubated, and unbound conjugated antibody is washed away. Finally, enzyme substrate (paranitrophenolphosphate-PNPP) is added, incubated, and the conversion of colorless PNPP to yellow paranitrophenol (PNP) product is measured in a spectrophotometer. The amount of substrate PNPP converted to product PNP is related to the amount of enzyme conjugated antibody bound which in turn is proportional to the amount of human anti-virus antibody bound to the immobilized antigen. Such an ELISA employing purified whole influenza virus as the antigen was chosen for the measurement of human specific antibody production in vitro. The ELISA which has been developed is rapid, sensitive and antigen specific. To assess specific anti-virus antibody production in vitro by human B lymphocytes peripheral blood mononuclear leukocytes are first washed extensively with tissue culture media to remove residual traces of serum antibody adherent to the cells. Then, mononuclear leukocytes or purified subpopulations of these leukocytes (T-cells, B-cells) are cultured in vitro with infectious allantoic fluid containing the antigenically distinct influenza viruses A/Hong Kong (A/Hong Kong/8/68-X-31 [H3N2]) or B/Hong Kong (B/Hong Kong/8/73); or with formalin-inactivited, zonally purified A/Aichi (A2/Aichi/68 MN 25241 [H3N2] and B/Hong Kong (B/HK/15/72 Rx 3560-1). After twelve days of in vitro culture, cumulative secretion of anti influenza A or anti-influenza B virus antibody into culture supernatants is detected by ELISA.

Major Findings: Cytotoxic T-cell Responses by Human Peripheral Blood Leukocytes in vitro: Studies were continued to define the cellular requirements for the in vitro generation of alloimmune and self-MHC restricted CTL, and the potential of cells from patients with immunodeficiency diseases to produce self-MHC restricted CTL. Previously, we had demonstrated that highly purified human T-cells were necessary and sufficient as responders in the production of alloimmune and self-MHC restricted CTL when stimulated with irradiated allogeneic leukocytes and irradiated virus infected autologous leukocytes, respectively. Additional studies were undertaken to define the requirement for monocytes in both cytotoxic systems. Peripheral blood mononuclear leukocytes containing T-cells, B-cells and monocytes were rigorously depleted of monocytes by sequential removal of cells capable of ingesting particulate iron and then cells possessing receptors for the chrystalyzable fragment (Fc) of immunoglobulin G. Such monocyte-depleted lymphocytes did not generate self MHC restricted CTL when influenza virus itself was added to these cultures but could generate virus specific CTL when mixed with irradiated adherent monocytes which themselves did not contain CTL precursors. Thus the in vitro generation of virus-specific self MHC restricted CTL was shown to be dependent

on both T-cells and monocytes. In additional studies, the presence of adherent monocytes bearing antigenic specificities recognized by the heteroantiserum anti-p 23,30 which recognizes human antigens similar to murine Ia (immune-associated) antigens were shown to be required for the generation of influenza specific CTL. When responder cells for the generation of alloimmune CTL were equally rigorously monocyte-depleted, the generation of CTL was diminished but not abolished. However, when both responder and the irradiated stimulator cells were monocyte-depleted no CTL were produced. The addition of irradiated adherent monocytes from either the responder or the stimulator individual restored the response even though neither population of monocytes contained CTL precursors. Thus the generation of human alloimmune CTL in mixed leukocyte cultures was dependent on monocyte function in the responder cells only when the irradiated stimulator population was monocyte-depleted. Since mixed leukocyte cultures and the generation of alloimmune CTL are routinely used for the assessment of immunocompetence in many patient studies, this observation is critical to the interpretation of these results. Monocyte defects within the responder cell population may only be observed when a stimulator cell population of peripheral blood leukocytes is devoid of monocytes. On the other hand, when virus immune CTL are produced by the direct addition of virus to peripheral blood mononuclear leukocytes, the absence of CTL production can result from T-cell and/or monocyte defects in the responder cell population.

The remainder of our studies of cytotoxic T-cell function focused on examining the capacity of peripheral blood leukocytes from immuno-deficiency disease patients to produce antigen-specific self-MHC restricted CTL. Given the observation that these CTL are restricted to recognize antigen and self-MHC gene products, one might ask how these receptor specificities are acquired? Studies of the cellular maturation processes and cell-cell interactions required for the production of virus-specific self-MHC restricted CTL in experimental animals have suggested the following scheme: 1) bone marrow stem cells differentiating into T-cells within the thymus develop receptor specificities for those MHC gene products expressed on the epithelium of the thymus prior to antigenic exposure, 2) these receptor specificities which then constitute the repertoire of MHC gene products considered as self are stable in the peripheral post thymic T-cell population, 3) T-cells expressing receptor specificities for foreign antigens plus self-MHC gene products are then selected from this peripheral Tcell pool when antigen is encountered in association with irradiation resistant lymphoreticular cells (? monocytes), and 4) the maturation of such antigen-specific self restricted CTL requires an interaction with a separate class of T-cells termed helper cells and may be negatively regulated by another T-cell subset termed T-suppressor cells.

Since results in experimental animals suggested that self-MHC restricted CTL play a major role in the recovery from viral infections and the lysis of autologous tumor cells, studies were undertaken to assess virus-specific self-HLA restricted responses in patients with immunodeficiency diseases who have frequent infections and an increased incidence of neoplasia. One such immunodeficiency disease is termed common variable hypogammaglobulinemia.

These patients have low levels of antibodies (gammaglobulins) in their serum and fail to make specific antibodies following in vivo antigenic challenge. In contrast to this observed functional B-cell defect, the patients exhibit a variable pattern of T-cell immunocompeteuce. Of eleven common variable hypogammaglobulinemic individuals studied thus far, the peripheral blood mononuclear leukocytes of nine patients have produced influenza virus-immune CTL capable of lysing autologous virus-infected target cells. Since the assay system employs virus immune CTL tested on infected autologous target cells, negative results could be caused by either defective CTL generation and/or target cells which did not express the relevent antigenic structures (HLA antigens, virus antigens) recognized by immune effecotrs. In the two patients in which virus-immune CTL for autologous targets were not detected, this lack of responsiveness was shown to be a defect in effector CTL generation since virus-infected target cells from both individuals were lysed by HLA compatible immune CTL from normal donors. Moreover, this deficiency within the effector cell population was not simply attributable to the lack of T-cells since both patients possessed normal proportions of T-cells within their cultured peripheral blood mononuclear leukocytes. Thus the majority of patients with common variable hypogammaglobulinemia possessed leukocytes capable of generating virus-immune, HLA restricted CTL; an experimental finding in accord with clinical observations that these patients are predominantly troubled by recurrent bacterial rather than viral infections.

Another human immunodeficiency disease studied for virus-specific CTL production in vitro was ataxia-telangiectasia. This disease is an autosomal recessive disorder characterized by progressive spinocerebellar degeneration leading to a clumsy gait (ataxia), oculocotaneous dilated blood vessels (telangiectasia), recurrent infectious, and a high incidence of neoplasia. Histopathologic examination of the thymus gland from ataxia-telangiectasia patients reveals a thymus which if fetal in appearance and almost totally devoid of epithelial elements. Since the aforementioned studies of the development of virus-immune CTL in experimental animals suggested a critical role for the thymic epithelium in the maturation of self-MHC receptor specificities on CTL precursors, we were particularly interested in whether cells from ataxia telangiectasia patients could produce antigen-specific self-HLA restricted CTL. Of the ten ataxia telangiectasia patients studied thus far, the mononuclear leukocytes nine have failed to produce significant lysis on autologous virus infected target cells. In five of these nine patients, this lack of CTL function was shown to be a defect in effector CTL production since virus-infected patients' cells weve lysed by normal HLA-related immune CTL. This defect was not readily attributable to lack of T-cells within the responder leukocytes since the proportion of T-cells among the patient's cultured leukocytes was not significantly different than normal. Additional studies were therefore undertaken to investigate the mechanism of this nonresponsiveness in ataxia-telangiectasia patients. The serum of nearly all ataxia-telangiectasia patients contains elevated levels of alpha-fetoprotein, a fetal protein not normally found in adult serum. Since immunosuppressive effects have been ascribed to alpha-fetoprotein, we considered the possibility that the non-responsiveness of these patients might be due to serum inhibitory effects. Therefore, normal peripheral

blood mononuclear leukocytes were cultured with virus in the presence of 5% normal pooled plasma (usual culture conditions), or 5% pooled plasma from ataxia-telangiectasia patients with elevated alpha-fetoprotein levels.

We found that normal cells generated the same virus-immune CTL activity in plasma from ataxia-telangiectasia patients as in normal plasma. Thus ataxia-telangiectasia plasma did not appear to exent marked immunosuppressive effects on normal cells and the non-responsiveness of patient's leukocytes was not easily attributable to plasma inhibitory effects. An additional mechanism which might account for the non-responsiveness of ataxia-telangiectasia leukocytes is the presence of excessive cell-mediated immunosuppression. In order to evaluate the potential of excessive suppressor cell activity, coculture experiments were undertaken in which cells from one ataxia-telangiectasia patient which did not produce virus immune CTL were mixed in an equal proportion with cells from an MHC identical normal sibling which were capable of producing virus-immune CTL. Such MHC-matched patient-sibling combinations are required for these experiments to avoid the generation of alloimmune CTL. If excessive cellular suppression was responsible for the non-responsiveness in the patient then this suppression might be expected to reduce the CTL activity generated by the sibling's cells. This experiment demonstrated that the MHC matched sibling's cells generated the same CTL activity whether or not they were co-cultured with the patient's cells. Thus in this one available patient-sibling combination, excessive cellular suppression did not appear to be the mechanism whereby leukocytes from the patient failed to generate self-MHC restricted CTL. The mechanism of this lack of responsiveness to viruses in association with self-HLA antigens in patients with ataxia-telangiectasia is still under investigation. Defective monocyte and/or T-helper cell functions remain to be tested. However, this unresponsiveness to viruses and self-MHC antigens may play a role in these patients undue susceptability to recurrent infections and neoplasia.

The third immunodeficiency disease studied for virus-immune self-MHC restricted CTL production in vitro is the Wiskott-Aldrich Syndrome. The Wiskott-Aldrich Syndrome is a sex-linked recessive disorder characterized by low peripheral blood platelet counts (thrombocytopenia) recurrent infections, a skin condition termed eczema, and an increased frequency of malignant neoplasms. Common viral infectious such as chickenpox may be life-threatening in these patients and in one case neoplasia was associated with persistent urinary excretion of a papova virus and the same virus was isolated from the patient's tumor. In seven Wiskott-Aldrich Syndrome patients studied thus far, the peripheral blood leukocytes of six failed to lyse influenza virus-infected, autologous target cells. The one patient who responded produced only minimal CTL activity which was greater than 16 fold less than simultaneously assayed normal controls. In all six patients not manifesting CTL activity, this defect was attributable to deficient production of immune CTL effectors since the virally infected patients' target cells were lysed by virus immune CTL from normal, HLA-related individuals. To investigate whether this defect in the production of immune CTL in the Wiskott-Aldrich Syndrome is limited to viruses, experiments have been initiated to investigate the capacity of patient's cells to mediate TNPspecific cytotoxicity. One

patient studied thus far also lacked peripheral blood leukocytes mediating TNP specific cytotoxicity. This non-responsiveness was attributable to defective CTL effector production since the patient's TNP modified target cells were lysed by TNP-immune, HLA-related normal CTL. Thus patients with the Wiskott-Aldrich syndrome are deficient in their ability to produce immune cytotoxic T-lymphocytes in vitro.

Recently, immunohematologic normalization has been reported in patients with the Wiskott-Aldrich Syndrome following bone marrow ablative therapy and transplantation of bone marrow from MHC identical siblings. One patient whose cells lacked the capacity to produce virus-immune CTL prior to such therapy generated normal influenza immune CTL activity six months following transplantation therapy which was performed by Drs. O'Reilly and Good at the Sloan-Kettering Institute. An additional Wiskott-Aldrich patient who received immuno suppressive therapy (without bone marrow ablation) and bone marrow transplantation from a MHC identical sibling, also possesses a normal potential to generate influenza immune CTL in vitro. While the precise mechanism(s) of non-responsiveness to viruses and self-MHC gene products in the Wiskott-Aldrich Syndrome remains unresolved, this defective production of virus-immune CTL is correctable by bone marrow transplantation following either immunosuppression or bone-marrow ablative therapy.

These studies of virus-immune self-MHC restricted CTL production demonstrate a heterogeneity in responsiveness among immunodeficiency disease patients. Most hypogammaglobulinemic patients but not patients with ataxia-telangiectasia on the Wiskott-Aldrich syndrome are capable of generating immune CTL in vitro. These defects in the production of immune CTL may contribute to the pathogenesis of recurrent infections and the high incidence of neoplasia in some of these patients.

Humoral Immune Responses by human peripheral blood leukocytes in vitro.

Studies were initiated to develop methods for the assessment of specific humoral antibody production by human peripheral blood mononuclear leukocytes in vitro. Antibody molecules belong to a class of serum proteins termed the gammaglobulins. Since these gammaglobulins can confer immunity they are often termed immunoglobulins (Ig) and several distinct sub-classes of immunoglobulins (IgM, IgG, IgA, IgD and IgE) are known to exist. Immunoglobulin secreting cells are derived from a class of leukocytes called B-cells which possess cell surface immunoglobulin as antigen receptors. Studies in experimental animals have show that transition of B-cells into immunoglobulin secreting cells is antigen-dependent, monocytedependent, and for most antigens requires the positive influence of T-helper cells and is negatively regulated by T-suppressor cells.

Much of our understanding of the maturation and immunoregulation of the human humoral immune response has derived from in vitro studies employing polyclonal activators such as pokeweed mitogen (PWM) and the Epstein-Barr virus which activate immunoglobulin secreting cells through receptors which are not antigen-specific. We have developed a method for studying antibody

production by human PBMC in vitro which is antigen-induced and does not require the presence of polyclonal activators. For these studies, we have again employed influenza viruses as antigens. Cultures of peripheral blood mononuclear cells from >95% of normal adult individuals produce specific anti-influenza virus antibody in vitro in the presence of type A influenza viruses. Antibody secretion requires de novo protein synthesis, begins about day 5 of culture and reaches maximal rates between days 5 and 7 of culture. Antibody synthesis can be induced by both live influenza type A and B viruses as infectious allantoic fluid or formalin inactivated, zonally purified type A and B viruses. Antibody generation was both antigen dependent and virus specific at the induction phase since: 1) cultures in media alone or stimulated with normal allantoic fluid roduced no antibody and 2) cultures stimulated with type A viruses produced anti-influenza A antibody but not anti-influenza B antibody and vice versa. The production of antibody was shown to require the cooperative interaction of T-cells, B-cells and monocytes in culture. This requirement for T helper cells capable of promoting the maturation of B-cells into antibody secreting cells was further investigated using a series of hybridoma derived monoclonal antibodies directed at predominantly distinct subsets of human T-cells. Using this methodology the human T-helper cell for anti-influenza antibody responses was shown to bear the specificity recognized by the monoclonal antibody OKT4 but not the specificity recognized by the monoclonal OKT8 which recognizes virus immune CTL effectors. Thus a subset of human T-cells comprising 40-60% of the total T-cell population was identified as the helper cell subset for specific antibody responses by B-cells in vitro.

Studies have been initiated to utilize this method to study maturational and immunoreguatory abnormalities in patients with immunodeficiency diseases. Three patients studied with X-linked hypogammaglobulinemia and isolated growth hormone deficiency failed to produce antibody in vitro in spite of having the capacity to mount a specific cytotoxic T-cell response to the same virus. In addition all 3 patients' cells could provide help to normal allogeneic B-cells and did not inhibit antibody production by cells from normal individuals. Thus these patients have an immune defect involving antibody secreting cells or their precursors. Of eleven hypogammaglobulinemic patients studied with a different disease termed common variable hypogammaglobulinemia (CVH), five made measurable antibody responses in vitro. Since CVH patients make specific antibody responses poorly in vivo, the finding of intact in vitro antibody responsiveness suggests that some patients have a host environmental abnormality as the cause of their hypogammglobulinemia which can be overcome in vitro.

Specific antibody responses were also sought in patients with ataxia-telangiectasia and the Wiscott-Aldrich syndrome who lacked influenza specific CTL responses. Six of seven patients with the Wiskott-Aldrich syndrome failed to produce specific antibody as did 4 of 5 patients with ataxia-telangiectasia. Further studies were undertaken to define the cellular basis of this non-responsiveness in three ataxia-telangiectasia patients. One of the patients was shown to have intact monocyte function since his irradiated adherent

cells reconstituted antibody responses in macrophage depleted cutures of his MHC matched sibling's cells. All three patients had immunocompetent B-cells since their cells produced antibody when: 1) stimulated with the polyclonal B-cell activator Epstein-Barr virus, and 2) provided T-cell help in the form of allogeneic irradiated T-cells. These results suggested a defect in T-helper cells as partial cause for the immunodeficiency in ataxia-telangiectasia. However, when T-cells from two patients were added to purified allogeneic B-cells both were capable of "helping" antibody synthesis. These results suggest the existence of two subsets of T-cells, one which can help allogeneic B-cells and the second which helps autologous B-cells and that ataxia-telangiectasia patients are lacking the latter subset.

Thus we developed a method to study specific antibody responses by human peripheral blood mononuclear cells in the absence of polyclonal activators. This assay in combination with assays of self-MHC restricted CTL function toward the same antigens will prove to be a powerful tool for investigating maturational and immunoregulatory events in humans.

Proposed Course: Studies will be continued to assess T-cell mediated specific cytotoxic responses in normal individuals and in patients with immunodeficiency. Studies in experimental animals and man have suggested that the T-cell subsets involved in the production of immune CTL (i.e. CTL precursor, T-helper cells, T-suppressor cells) may be separable on the basis of cell surface antigens. Recently, methods have been developed whereby antibodies recognizing only sub-populations of human peripheral blood T-cells can be produced (hybridoma antibodies). Such antibodies will be tested for their ability to recognize T-cell subsets in normal individuals which are required for the generation of immune CTL and also to identify immune CTL effectors. If antibodies useful in dissecting CTL responses in normal individuals are found, these reagents will be used to further probe the mehanisms(s) of defective specific CTL responses in immunodeficiency diseases (i.e. lack of CTL precursors, T-helper cells or monocytes, excessive T-suppressor cells). Attempts will be made to assess hapten-specific CTL responses generated in vivo by immunization with the cutaneous application of dinitrochlorobenzene. Additional studies of hapten-specific CTL generation in vitro will be undertaken in patients with the Wiskott-Aldrich Syndrome and ataxia-telangiectasia to further assess the scope of defective CTL production in these individuals.

With regard to humoral antibody responses in vitro, studies will be expanded to further define the proportion of normal individuals capable of responding with antibody production to the influenza viruses and other antigens and the optimal in vitro culture conditions for generating such responses. The aforementioned studies employing hybridoma antibodies recognizing T-cells and additional antibodies possibly recognizing monocytes and B-cells will be expanded in attempts to further define the cellular interactions necessary to generate a response. Using purified virus antigens (i.e. virus hemagglutinin, virus neuraminidase), the specificity of the in vitro secreted antibody will be further defined. Attempts will be made to establish T-cell lines which may be cloned to proved purified subsets of T-helper and T-

suppressor cells for further analysis. Studies of specific humoral immune responsiveness in vitro will be continued in patients with immunodeficiency diseases, particularly those patient groups already defined as having defective specific CTL responses (ataxia telangiectasia, Wiskott-Aldrich syndrome) and those hypogammaglobulinemic patients with normal CTL responses. Specific CTL responses and specific antibody responses will then be studied in parallel to further elucidate the mechanism(s) involved in these patients susceptibility to recurrent infections and cancer.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-04016-09 MET

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Mechanism of the Anabolic Action of Growth Hormone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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5	3	2

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rat embryo fibroblasts have been shown to have receptors for MSA (multiplication stimulating activity), to respond to MSA (DNA synthesis), and to produce MSA the concentration of MSA in medium conditioned by the rat embryo fibroblasts approaches the concentration of MSA in fetal rat serum. Fibroblasts from skin and kidney of the pygmy mouse (pg/pg) have been shown to have receptors for MSA with characteristics indistinguishable from MSA receptors on fibroblasts from normal littermates.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The understanding of normal growth may be a prerequisite to understanding malignant growth. One experimental model for the study of normal growth is the growth hormone deficient animal following treatment with growth hormone. The anabolic effects of growth hormone are thought to be mediated by a second hormone called somatomedin. Somatomedin is a member of a family of peptides called insulin-like growth factors. The object of the project is to purify and characterize one of the insulin like growth factors called multiplication stimulating activity (MSA), study the regulation of the production of MSA, develop cell culture systems in which to study the biochemical events accompanying growth stimulated by MSA.

Methods Employed: MSA is being purified from serum-free medium conditioned by a rat liver cell line (BRL-3A) using ion exchange chromatography, gel filtration, and high pressure liquid chromatography (HPLC). Tertiary cultures of rat embryo fibroblasts derived from 16 day embryos are being used to study response to MSA receptors for MSA and production of MSA. Biologic response to MSA in rat embryo fibroblasts is being assessed by measuring cell multiplication and DNA synthesis (autoradiography with [^{3}H]thymidine). Cell surface receptors for MSA are identified using radioiodinated MSA. The MSA produced by the rat embryo fibroblasts is characterized using a specific MSA radioimmunoassay, radioreceptor assays, competitive protein binding assay, and bioassay ([^{3}H]thymidine incorporation into DNA in chick embryo fibroblasts). Fibroblasts are cultured from skin and kidney of pygmy mice (pg/pg) and normal littermates. These fibroblasts were characterized for response to MSA (cell multiplication and [^{3}H]thymidine incorporation into DNA) and MSA binding.

Major Findings: We recently reported that MSA is found in high concentration in fetal rat serum and declined to reach very low levels by day 20 of extrauterine life. This observation led to the proposal that MSA may be a fetal growth factor. Therefore, we were interested to study the effect of MSA in fetal rat cells in culture. We found that tertiary cultures of rat embryo fibroblasts derived from 16 day embryos have specific receptors for MSA. In contrast to MSA receptors in chick embryo fibroblasts and human skin fibroblasts where insulin competes for ^{125}I-MSA binding, insulin did not compete for ^{125}I-MSA binding to the rat embryo fibroblasts. The rat embryo fibroblasts did not respond to MSA alone (cell multiplication and DNA synthesis) but when added to platelet derived growth factor (PDGF) and platelet poor plasma (PPP) from growth hormone deficient rats (hypox), MSA stimulated DNA synthesis as measured by autoradiography. The combination of MSA, PDGF and hypox PPP produced the same response as a combination of PDGF and PPP from normal rats or normal rat serum. On the other hand, hypox serum or a combination of PDGF and hypox PPP produced only minimal DNA synthesis. Surprisingly, when a 4 day cell multiplication experiment was performed there was no difference in the growth rate of cells maintained in medium containing normal rat serum or hypox rat serum. One explanation for this result

would be that the rat embryo fibroblasts were producing MSA and during the 4 day experiment this MSA produced by the rat embryo fibroblasts together with hypox rat serum was as effective as normal rat serum in causing the cells to multiply. Indeed, when rat embryo fibroblasts were maintained in culture under serum-free conditions and the conditioned medium harvested, MSA was detected in this medium by MSA radioimmunoassay. The concentration of MSA in the medium after 3 days of culture approached the concentration of MSA in fetal rat serum. Cycloheximide blocked the production of MSA by the rat embryo fibroblasts showing that MSA was actually being synthesized. The MSA produced by the rat embryo fibroblasts was shown to be indistingushiable from MSA produced by the rat liver cell line BRL-3A in radioreceptor assays (chick embryo fibroblasts, chondrosarcoma chondrocytes, and rat liver membranes), a competitive protein binding assay (rat serum binding protein) and bioassay ([^{3}H]thymidine incorporation, chick embryo fibroblasts). Importantly, the rat embryo fibroblast MSA also stimulated the rat embryo fibroblasts to synthesize DNA when added to a combination of PDGF and hypox PPP.

The pygmy (pg/pg) mouse has been proposed as an animal model of growth hormone resistance because treatment of the pygmy mouse with large amounts of growth hormone did not stimulate growth. We showed subsequently that serum somatomedin activity measured by bioassay was normal in the pygmy mouse raising the possibility that the genetic defect in this mouse was distal to somatomedin production, either at the level of the somatomedin receptor or beyond. To investigate this possibility we cultured fibroblasts from skin and kidney of the pygmy mouse and normal littermates. We determined the growth (cell multiplication and [^{3}H]thymidine incorporation into DNA) of the mouse fibroblasts in response to combinations of PDGF, hypox PPP and MSA and also attempted to characterize an MSA receptor with respect to binding affinity for MSA and receptor number. We were not able to detect significant differences between the pygmy and normal fibroblasts for any of these parameters.

Mouse embryonal teratocarcinoma cells have been shown to differentiate in cell culture to form the many types of tissue making up the teratocarcinoma in animals. Because of the fetal origin of these cells it was of interest to examine the response of these cells to MSA. In collaboration with Wayne Anderson and Lalitha Nagarigan in the Laboratory of Pathophysiology it was found that MSA stimulated the multiplication of F9cells, an undifferentiated teratocarcinoma cell line. An MSA receptor was demonstrated on these cells. Insulin was also a mitogen for these cells but did not interact with the MSA receptor suggesting that insulin was stimulating multiplication of the F_9 cells by interacting with an insulin receptor. Earlier studies performed in collaboration with Matthew Rechler, NIAMDD, had demonstrated that insulin-like growth factor receptors were of two types; one which also interacted with insulin and a second which was insulin insensitive. Thus, in a rat liver cell line (BRL 3A-2) ^{125}I-MSA bound to a receptor which was insulin insensitive while ^{125}I-IGF-I bound to a receptor which also interacted with insulin. Recently a collaborative study with Masato Kasuga and Matthew Rechler, NIAMDD, has provided structural evidence for two different types of receptors. Masato Kasya chemically cross-linked ^{125}I-MSA and ^{125}I-IGF-I to receptors on the BRL 3A2 cells

and then solulizing the membranes and analyzed the radioligand receptor complexes on SDS disc acrylamide gels. He found that following reduction of disulfide bonds the ^{125}I-MSA receptor complex was a 260,000 dalton species while the insulin sensitive ^{125}I-IGF-I receptor complex was 130,000 daltons.

Significance to Biomedical Research:

The finding that rat embryo fibroblasts produce MSA, have receptors for MSA and respond to MSA shows that the regulation of growth of normal cells can in part be due to growth factors produced by cells in the same population. The same observation has been made by others for human fibroblasts and somatomedin C and transformed cells have been shown to produce factors which can lead to transformation of normal counterparts of the same cells. The failure to detect abnormalities in the mouse pygmy (pg/pg) fibroblasts either for MSA binding or response to MSA suggest that the genetic defect is tissue specific or that the genetic defect in the pygmy mouse is outside the growth hormone/somatomedin pathway., i.e., the pygmy mouse is not a model for peripheral resistance to growth hormone or somatomedin.

Proposed Course:

Cells in culture derived from other tissues in the rat embryo will be examined to see whether they also produce MSA and respond to MSA. We will study the regulation of MSA production by the rat embryo fibroblasts using growth hormone and placental lactogen. We will attempt to develop monoclonal antibodies to the MSA receptor of the chondrosarcoma chondrocyte. Such antibodies will be used to attempt to provide further evidence for functional importance of the MSA receptor. We will continue to examine skin fibroblasts from patients with short stature who are candidates for having and organ resistance to somatomedin.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-04004-21-MET

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies of Amino Acid Biosynthesis and Degradation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James M. Phang, M.D.	Senior Investigator	MET	NCI
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COOPERATING UNITS (if any)

David Valle, M.D. - John Hopkins University School of Medicine
Robert J. Smith, M.D. - Joslin Research Laboratories

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

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6	3	3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The enzymes catalyzing the interconversion of proline and pyrroline-5-carboxylate provide a mechanism for the intercompartmental, intercellular, and interorgan transfer of redox potential. Mediated by the transfer of redox potential, PC regulates PPRP and nucleotide production thereby linking amino acid and nucleotide metabolism.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Recent reports have emphasized the primary role of metabolic regulation in malignant transformations of mammalian cells. The transport, biosynthesis and degradation of amino acids may play an important role in this metabolic regulation. Not only are amino acids the necessary building blocks for proteins but also they function as regulators in intermediary metabolism. This project emphasizes the biosynthesis and degradation of proline because the metabolism of proline has unusual, even unique, features. The metabolic intermediates of proline provide an important link of carbons between the TCA and urea cycles. In addition, these intermediates, functioning as redox couples, can regulate a number of redox-dependent metabolic pathways e.g., the production of nucleic acids. These studies may elucidate changes in growth rates, energy allocation and malignant transformations in tumors. The project is relevant to objective 3, Approaches 1 and 5 of the National Cancer Program.

Methods Employed: A variety of approaches are used to pursue the aforementioned objectives in animal tissues, freshly isolated human cells and cells maintained in long-term tissue culture. Cultured cell lines are especially useful since they can be cloned to insure genetically homogenous populations. Mutant cells are isolated by standard techniques or are obtained from patients with defined inborn errors of metabolism. Many biochemical techniques are utilized in these studies. Uptake of amino acids are assessed by using labeled amino acids and rates of protein and collagen synthesis are obtained by incubating cells with L-proline-^{14}C and separation of incorporated labeled amino acids by ion-exchange column chromatography. Metabolic conversions in intact cells can be determined by isolating labeled products from labeled precursors using high-pressure liquid chromatography. Methods have been developed to measure ornithine production from proline and glucose formation from lactate, proline and alanine. Nucleotides and their precursors, labeled and unlabeled, are quantitated using high-pressure liquid chromatography. The enzymes of biosynthesis and degradation of proline are assayed using specific radioisotopic methods. Biochemical studies on specific enzymes include the isolation of mitochondria, solubilization of enzymes from mitochondrial particles and purification. Physicochemical characteristics of these enzymes are studied by gel filtration and affinity chromatographic techniques.

Major Findings:

Clinical and Basic Research Goals: Proline, a nonessential amino acid, has a number of unique metabolic functions. Proline and its hydroxylated derivative, 4-hydroxy-L-proline, are abundant in collagen constituting 20% of the amino acid residues. More interestingly, our laboratory has shown that proline and its metabolism can regulate other major metabolic pathways thereby providing a mechanism for modulating the use of alternative energy sources. The two imino acids (PRO, HOP) are unusual in that they are the only naturally occuring amino acids without a free alpha-amino group. Instead, the alpha-amino group is incorporated within a pyrrolidine ring. Thus, proline and hydroxyproline

cannot participate in the usual transamination and decarboxylation reactions common to most other amino acids. We have shown that the specific enzyme system metabolizing proline is linked to general metabolic pathways via the redox state. Since the redox state is critical to a large number of metabolic systems (energy, gluconeogenesis, nucleotide metabolism, membrane function, ureogenesis, etc.) we propose that proline plays a role in regulating these metabolic systems and derangements of this regulation may produce disease.

The Proline Cycle: We have proposed that the enzymes catalyzing the interconversions of proline, pyrroline-5-carboxylate (PC), ornithine and glutamate constitute a metabolic system for the intercompartmental, intercellular and interorgan transfer of redox potential. Central to this hypothesis is the proline cycle in which proline and pyrroline-5-carboxylic acid (PC) function as a redox pair and their interconversions catalyzed by proline oxidase and pyrroline-5-caraboxylate reductase, mediate the redox transfers. PC reductase, a cytosolic enzyme, oxidizes either NADH or NADPH accompanying the formation of proline. Proline oxidase which is tightly bound to mitochondrial inner membranes converts proline back to PC, donates protons to electron transport and thereby phosphorylates ADP by a mechanism independent of NADH oxidation. The availability of PC for participation in the proline-PC cycle may depend on the enzymes catalyzing the interconversions of ornithine and glutamate, i.e. PC synthase, ornithine aminotransferase and PC dehydrogenase; all of which have PC either as substrate or product and thereby determine the availability of PC for participation in the proline-PC cycle.

Direct Demonstration of Proline Cycling

Our previous studies on proline cycling emphasized the metabolic effects of the cycle on the oxidation of glucose through the hexosemonophosphate-pentose pathway. Additional evidence supporting the proline cycle has come from the direct demonstration of one turn of the proline cycle. Using glucose-(1)-^{3}H, $NADP^{+}$, PC, hexokinase, glucose-6-phosphate dehydrogenase and PC reductase in the presence of rat kidney mitochondria, we showed that proline added to the incubation became tritiated. The accumulation of tritium into proline was dependent on duration of incubation and on the amount of mitochondria particles. Accumulation of tritium in proline occurred because proline was oxidized to PC by mitochondria and reconverted back to proline by PC reductase. The incorporation of tritium occurred in the PC reductase reaction with glucose-(1)-^{3}H as the source and $NADP^{3}H$ as the transferring intermediate. These studies clearly show that the proline cycle can transfer redox between mitochondria and cytosol in an *in vitro* system.

Physiologic Effect of PC: Nucleotide Metabolism in Human Erythrocytes

We have shown that pyrroline-5-carboxylate (PC), the central intermediate in the interconversions of proline, ornithine and glutamate, can be transferred into human erythrocytes as functioning oxidizing potential. PC markedly increases the flux of glucose through the hexosemonophosphatepentose (HMP) pathway. The mechanism for the effect is due to the PC dependent oxidation of

NADPH. Since the HMP pathway is an important source of pentose and phosphoribosyl pyrophosphate (PPRP), we considered that PC would stimulate PPRP synthesis and nucleotide formation from purines via the salvage pathway in erythrocytes. We first measured PPRP concentrations in human erythrocytes using a coupled assay in which the generation of $^{14}CO_2$ from [carboxyl-^{14}C]orotic acid in the presence of orotic acid pyrophosphorylase and orotidine decarboxylase is quantitatively dependent on the amount of PPRP. In studies using erythrocytes from 6 normal subjects, we found that a 20 minute incubation with PC markedly increased PPRP levels. PPRP levels in incubated controls were 10.1 $\pm$ 1.4 nmol/ml cells and in PC treated cells were 79.5 $\pm$ 14.5 nmol/ml cells. The activity of the HMP pathway in parallel incubations increased from 152 $\pm$ 19 nmol/h-ml cells in controls to 772 $\pm$ 41 nmol/h-ml cells with PC. The increase in PPRP concentration was accompanied by an increased incorporation of purines into nucleotides by the salvage pathway. With PC, the incorporation of ^{14}C-labeled adenine, guanine and hypoxanthine into their respective nucleotides increased 3.0, 2.2 and 2.5 fold, respectively. Since PC stimulated the flux through the salvage pathway, the metabolic fate of adenosine was of special interest since this ribonucleoside can be taken-up by erythrocytes and either directly phosphorylated by a kinase or sequentially converted to inosine, hypoxanthine and IMP by the salvage pathway. We found that adenosine incorporation into adenine nucleotides was unaffected by PC-treatment whereas the incorporation of adenosine into IMP was increased by PC. Thus, in erythrocytes, it is the salvage pathway, a PPRP-dependent pathway which is stimulated by PC. Therefore, PC, through its effects on PPRP, can regulate an important branchpoint in nucleotide metabolism.

The Regulation of PC Synthase

In collaboration with Dr. Robert J. Smith of the Joslin Research Laboratories, we developed an assay for PC synthase activity, the reaction catalyzed by a multi-enzyme complex which converts glutamate to pyrroline-5-carboxylate. We found that ornithine directly and reversibly inhibits PC synthase activity. The apparent K_i for ornithine is 0.16 mM. Physiologic validation of the regulation by ornithine was established in cultured fibroblasts from patients with gyrate atrophy. These cells lack ornithine aminotransferase activity and cannot convert ornithine to PC. In these cells, ornithine added to the medium, markedly decreased the quantity of glutamate-derived proline incorporated into proteins. These findings suggest that ornithine is the preferred precursor for PC and proline and that when ornithine is available, the conversion of glutamate to proline is inhibited.

Ornithine Toxicity in GA Cells - Relationship to PC Deficiency

Gyrate atrophy of the choroid and retina is an autosomal recessive inherited disease with progressive retinal degeneration resulting in blindness by the 5th decade. Although the enzymatic defect has been identified as a deficiency in ornithine aminotransferase, the pathophysiologic mechanism producing blindness is not understood. In collaboration with David Valle of Johns Hopkins University School of Medicine and Robert J. Smith of the Joslin Research Laboratories, we used ornithine toxicity in these cells as an experimental model. Ornithine is toxic but only to gyrate

atrophy fibroblasts. This finding together with the aforementioned inhibitory effect of ornithine on PC synthase suggested that gyrate atrophy cells are adversely affected by ornithine because they cannot produce PC from ornithine and ornithine inhibits the production of PC from glutamate. Normal cells, in contrast, are not sensitive to ornithine toxicity because they can produce PC from ornithine even though the production of PC from glutamate is inhibited. These studies suggest that ornithine toxicity in gyrate atrophy cells is due to a deficiency in PC production. The pathophysiologic mechanism of PC deficiency may relate to the regulatory effects of PC on PPRP and nucleotide production.

Significance to Biomedical Research

The linkage of the proline metabolic pathway to glucose (energy) and nucleotide metabolism has far-reaching implications. Since the HMP pathway is the source of pentose for nucleic acids, the proline-PC interconversions provide a mechanism for adjusting the synthesis of macromolecules. Derangements in the linkage may be involved in the altered metabolism of malignant cells. Furthermore, the demonstrated proline-PC cycle provides not only a mechanism for ATP generation from the HMP pathway, but also serves as a model for small molecules acting as metabolic signals between cells. These studies provide a new approach in understanding pathophysiologic mechanisms in several diseases including: 1) metabolic derangements in sepsis, 2) increased collagen and proteoglycan production in diabetes melltius, 3) retinal degeneration in gyrate atrophy and, 4) oncogenesis.

Proposed Course:

Recent studies on the mechanisms of oncogenesis (Src gene, etc.) have re-emphasized the importance of studies in the area of metabolic regulation. We think that the regulatory effects of the PC-proline axis is an important new facet of metabolic regulation. During the coming year we will emphasize the following specific areas:

1) The demonstration of catalytic cycling of proline-PC in intact cells and in an *in vitro* system. Although we have shown one complete turn of the proline cycle, the catalytic cycling of proline and its effects have not been demonstrated. Using proline radiolabeled with both ^{14}C and ^{3}H in the 5 position, we hope to show that tritium transfer into $^{3}H_2O$ will be linear with time but diappearance of ^{14}C from PRO-PC will plateau. Stoichiometric correlation between $^{3}H_2O$ and NADPH oxidation will establish the catalytic nature of the proline cycle. Using a similar approach we hope to show that this can occur in intact cells.

2) The effects of PC on nucleotide metabolism. We hope to extend our understanding of PC as a regulator of nucleotide metabolism in cells undergoing activation. Lymphocyte activated by mitogens may be an useful experimental system. We will study the PC effects on PPRP synthesis, *de novo* synthesis of nucleotides and their incorporation into macromolecules.

3) Pathophysiologic mechanisms in gyrate atrophy cells. We will emphasize the function of PC in these cells which have demonstrated deficiency in PC production. We hope to establish that the deficiency in PC results in abnormalities in PPRP synthesis and nucleotide metabolism.

4) Protein chemistry of PC reductase. We previously showed that PC reductase is present at high levels in erythrocytes and the enzyme in these cells has kinetics characteristics and regulatory features distinct from the enzyme found in other tissues. Since these findings suggest the existence of isozymes of PC reductase, we hope to identify isozymes by physiochemical physicochemical methods. Purification of the enzyme from erythrocytes may provide suitable substrate for studies directed at identifying possible post-translational modification (phosphorylation, etc.).

5) Demonstration of the regulatory effects of PC in a physiologic system. With our insights into PC effects on erythrocytes, we hope to study PRO/Re mice which have deficient proline oxidase and use nucleotide metabolism in their erythrocytes as a metabolic endpoint. Other investigators have used intraperitoneal injections of proline to introduce a hepatic proline load. We hope to use similar approaches of proline delivery and use the red cell in post-hepatic blood as a measure of the intercellular transfer of oxidizing potential.

6) A specific assay for PC levels in cells and tissue. Although the studies of the PRO-PC regulatory axis has been established, possible cellular fluctuations in PC have not been studied. We hope to develope an assay using derivatized PC and High-pressure liquid chromatography which would have enough sensitivity to measure PC concentrations in cultured and circulating cells.

7) Viral transformation and the enzymes of proline metabolism and the PRO-PC regulatory axis. Although we have previously demonstrated differences in levels of activity of the proline metabolic enzymes between control and transformed cells, we were unable to directly relate the changes in enzyme activity to the transformation process. We hope to use cells transformed with temperature-sensitive mutants of SV40 virus to establish this relationship.

Publications:

Smith, R.J., Downing, S.J., Phang, J.M., Lodato, R.F., and Aoki, T.T.: Pyrroline-5-carboxylate synthase activity in mammalian cells. Proc. Natl. Acad. Sci. USA. 77: 5221-5225, 1980.

Phang, J.M., Yeh, G.C., and Hagedorn, C.H.: The intercellular proline cycle. Life Sciences. 28: 53-58, 1981.

Yeh, G.C., Harris, S.C., and Phang, J.M.: Pyrroline-5-carboxylate reductase in human erythrocytes. A comparison of differential regulation. J. Clin. Invest. 67: 1042-1046, 1981.

Lodato, R.F., Smith, R.J., Valle, D., Phang, J.M., and Aoki, T.T.: Regulation of proline biosynthesis: the inhibition of pyrroline-5-carboxylate synthase activity by ornithine. Metabolism. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-04014-11-MET

PERIOD COVERED

October 1, 1980 through September 30, 1981.

TITLE OF PROJECT (80 characters or less)

Studies of the Immune Response in Normal and Pathological States

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Steven James, M.D.	Investigator	MET	NCI
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COOPERATING UNITS (if any)

LAB/BRANCH

Metabolism Branch

SECTION

Immunophysiology Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	4	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were directed toward the analysis of immune response in normal humans and patients with a variety of immune disorders, as well as analysis of humoral and cellular immune responses of experimental animals.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Studies were directed toward the analysis of the humoral and cellular immune responses of experimental animals with particular emphasis on the study of experimental models of human disease. In addition, studies were directed toward the analysis of the immune responses of normal humans as well as individuals having a variety of immune disorders with concentration on immunodeficiency disorders as well as immunologic disorders of the gastrointestinal tract. These studies included the investigation of patient with glutensensitive enteropathy, dermatitis herpetiformis, inflammatory bowel disease, as well as patients with ataxia telangiectasia, common variable hypogammaglobulinemia, and intestinal lymphangiectasia. Each of these diseasea are associated with a high incidence of reticulo-endothelial malignancy and the mechanism underlying the association between malignancy and these diseases was sought.

Methods Employed: A host of techniques were used to assess immunologic function of experimental animals and patients with disorders of the immune system. Circulating B cells in peripheral blood and in tissue were enumerated with carefully prepared fluorescein-conjugated F(ab')2 antiserum with specificity for heavy chains of immunoglobulins. Circulating T cells were enumerated with sheep red blood cell rosette techniques. Antibody responses were assessed by microhemagglutination techniques and in vitro proliferative responses were assessed with tritiated thymidine incorporation following exposure to specific and nonspecific mitogens. Mitogen-induced cytotoxic effector cells as well as antibody dependent and natural cytotoxic effector cells were assessed with ^{51}Cr-release assays using red cell and nucleated, metabolically active target cells. Homogenous cell populations were isolated with the use of Sephadex anti-$F(ab')_2$ columns, rosetting techniques and the use of colloidal iron followed by passage through a magnetic field. Short term organ culture was used in conjunction with solid phase immunoabsorption techniques to measure mucosal immunoglobulins; long term organ culture techniques were used to study in vitro models of intestinal disease.. Cells obtained from various tissues as well as from peripheral blood were cultured in vitro under a variety of conditions. Supernatants from these cell cultures were assayed by radioimmunoassay in order to measure immunoglobulins produced in culture. Cells were adapted and maintained in long term culture with the use of T cell growth factors. Patients with immunologic disorders were studied with metabolic turnover techniques to measure immuoglobulin synthesis and catabolism.

Major Findings:

A. STUDIES OF THE REGULATION OF IgA IMMUNOGLOBULIN RESPONSE

Backgound: In recent years we have been concerned with the cellular mechanisms regulating mucosal immune responses in general and IgA immunoglobulin responses in particular. In this regard, we have demonstrated that IgA immunoglobulin synthesis are governed by regulatory T cells which are distinct from the regulatory T cells controlling IgM and IgG synthesis. First we showed that lipopolysaccharide (LPS)-induced Ig

synthesis by indicator mouse B cells was variably affected by Concanavalin A (Con A) treated Peyer's patch T cells: IgM and IgG synthesis was suppressed whereas IgA synthesis was enhanced. Next we showed that feeding a protein antigen (BSA) to mice resulted in the appearance in Peyer's patches of antigen-specific suppressor T cells for IgG anti-BSA responses concomitant with the appearance of antigen-specific helper T cells for IgA anti-BSA responses. This was shown in cell transfer studies using fed animals as donors of regulatory T cells and parenterally stimulated animals as recipients to the regulatory T cells. In this system it was also possible to show with monoclonal anti-LyT-2 (anti-suppressor T cells antibody) that antigen-specific suppression for the IgG response could be affected without affecting antigen-specific help for the IgA response and vice versa. Thus, these prior studies provided data from both in both polyclonal and antigendriven systems that the regulation of IgA responses was independent of the IgG/IgM response.

During the current period we have extended this line of investigation by investigating the properties of cloned regulatory T cells maintained in continuous culture with interleukin 2 (IL-2) a T cell growth factor. In these studies we first established non-cloned Concanavalin A-stimulated T cells obtained from small intestinal Peyer's patches, colonic Peyer's patches and spleens of Balb/c mice. When such cells were able to survive in culture with periodic replacement of IL-2 (but without re-stimulation with Con A) we cloned each of them by limiting dilution and ultimately obtained two Peyer's patch T cell lines (obtained from separate cell sources), two spleen T cell lines (also from separate cell sources) and one colonic Peyer's patch T cell line. Clonal frequency analysis determined by limiting dilution and Poisson distribution indicated that each clone had in fact originated from a single cell. Analysis of re-cloned cells supported this conclusion.

The characteristics of these cloned lines was then tested with the following results: 1) the cloned T cells all bear Thy 1.2, LyT 1.2 and Ia ($I\text{-}A^d$ and $I\text{-}E^d$) as well as H-2 ($H\text{-}2^d$ K/0) surface antigens; the cells are non-specific esterase negative, non-phagocytic and surface and cytoplasmic Ig negative; finally, the cells are non-adherent to nylon-wool columns; 2) the cloned T cells can be stimulated to proliferate by Con A or PHA, but not LPS; 3) the cloned T cells have no capacity to lyse eight types of syngeneic, allogeneic and xenogeneic targets, i.e., are not cytotoxic effector cells; 4) the cloned cells cannot mediate ADCC and 5) the cloned T cells have an interesting distribution of Fc receptors: approximately 60% of the cells from each Peyer's patch derived clones bear IgA-Fc receptors whereas none of the spleen-derived clones or colonic Peyer's patch clone bears this receptor. Some of the cells in the Peyer's patch-derived clones also bear IgG-Fc receptors (40%) as well as IgM-Fc receptors (1-2%). The cells in the spleen and colonic Peyer's patch clone bear only IgG-Fc receptors when they bear any receptor at all.

The ability of the cloned cells to produce IL-2 was tested and it was found that when stimulated with Con A or PHA each cell line produced

this growth factor. This production was inhibited by irradiation of cells and treatment with hydrocortisone.

Having characterized the cloned cells in this manner we proceeded to test their capability to serve as helper cells for inducing production of IgA and other immunoglobulins. We found that each of the cloned T cell, regardless of their origin (and thus regardless of their IgA-Fc receptor status, was capable of helping B cells produce IgA. Thus IgA-Fc receptors are not necessary to IgA synthesis. In preliminary studies we also tested the capacity of cloned T cells to initiate suppression of Ig synthesis and found that when such cells are mixed with fresh T cells they do induce suppressor cell activity. However, the class specificity of this suppressor cell activity is not yet known.

Finally, we found that the cloned T cells produced supernatant materials which had the capability of helping LPS-induced IgA synthesis by purified B cells. Thus, it was apparent that a humoral factor could direct B cell differentiation along a pathway that leads toward IgA synthesis (and, presumably away from exclusive IgM synthesis).

Significance: The significance of this work is that for the first time we can measure directly the function of IgA-Fc receptor function. Clearly, this receptor has little or no affect on the helper aspect of T cell activity. Nevertheless, the receptor may yet be intimately involved in class-specific immunoglobulin suppression and these cloned cells will, in fact, enable us to investigate this question. Additionally, these cloned cells allow us to study the function of humoral factors produced by LyT-1 helper cells having defined characteristics.

Proposed Course:

1) Most importantly we plan to study the capacity of the above described cloned cells to induce class-specific suppressor cells. As alluded to above, this will be done by mixing fresh T cells with cloned cells and studying the subsequent capacity of the fresh T cells to suppress LPS-induced Ig synthesis of various immunoglobulins. The latter will be assayed by radioimmunoassay or reverse hemolytic plaque technique.

2) We have accumulated some evidence that the non-specific growth factors produced by the cloned cells involve several different molecules each acting on separate cell types. We plan to explore the possibility that separate non-specific growth factors act on LyT-1 helper cells and LyT-1 cytotoxic/suppressor cells.

3) We have begun to study the capacity of syngeneic and allogeneic cells to activate the cloned T cells and induce them to proliferate. In preliminary studies we have found that the cloned cells respond to both allogeneic and syngeneic B cells and macrophages. This interesting and surprising result will be explored with the use of monoclonal anti-Ia antibodies in an attempt to elucidate the relevant antigens in this kind of stimulation.

4) As a new departure, we are in the process of establishing antigen-specific T cell helper and suppressor T cell clones in order to compare results obtained with cell clones obtrained from polyclonal activation with those obtained with antigen activation.

STUDIES OF IMMUNOREGULATORY DEFECTS IN CROHN'S DISEASE

Background: Crohn's disease is an inflammatory disease of the small bowel and/or large bowel characterized by ulceration and granuloma formation. Patients develope bowel wall thickening, fistula formation as well as extra-intestinal manifestations including arthritis, iritis and skin lesions. In previous studies of immune function of patients with Crohn's disease, a number of abnormalities have been identified, but none which could lay claim as the primary cause of the disease.

In prior studies of Crohn's disease (CD) we have explored the possibility that this disease results from or is complicated by an abnormality in the regulation of the immune response. More specifically we have shown that many CD patients with relatively mild or inactive disease have in their peripheral blood a "covert" suppressor T cell which exerts profound suppressor cell activity detectable in vitro by the use of indicator cultures consisting of allogeneic purified B cells and irradiated T cells driven to produce Ig by the polyclonal stimulant pokeweed mitogen (PWM). Of great interest was the fact that the suppressor T cell was not manifest in unseparated cell populations, but only after purification of T cells on Sephadex-anti-F(ab')$_2$ columns; hence the term "covert" suppressor T cells.

During this period we showed that the covert suppressor T cells could be active in vivo as well as in vitro. In these studies we investigated several patients who had both Crohn's disease as well as hypogammaglobulinemia. First, we showed that these patients' peripheral blood contained overt suppressor T cells which were manifest prior to cell separation on anti-F(ab')$_2$ columns as well as after cell separation. Second, we showed that these patients' B cells were capable of PWM-driven Ig synthesis providing allogeneic normal T cells were added to the cultures of the patient's B cells. More importantly, we showed that these patients' B cells were stimulated to produce Ig when a stimulate not requiring T cell help, EB virus was added to the culture. Thus, there was no defect in the patients' B cells and the hypogammaglobulinemia could indeed be attributed to an overt suppressor T cell.

The existence of patients with Crohn's disease and hypogammaglobulnemia raises the question of why in most patients the suppressor T cells are covert and there is no suppression of Ig synthesis in vivo whereas in very occasional patients such cells are overt and suppression of Ig synthesis in vivo is seen. The answer to this lies in as yet incomplete studies wherein it has been shown that the T cell populations in Crohn's disease is, in reality, a complex mixture of regulatory cells which includes cells which counteract the action of suppressor T cells, anti-suppressor cells. This conclusion is drawn from the fact that in several

studies we have shown that separation of T cells on anti-$F(ab')_2$ columns results in an initial T cell fraction which contains the suppressor cells noted in CD patients and a second, more adherent fraction which contains cells which oppose suppressor cell activity without providing helper activity. On the basis of these studies we speculate that hypogammaglobulinemia can occur in Crohn's disease when suppressor T cells are unopposed by other cells which occur along with or are induced by the suppressor cells. In other words, the hypogammaglobulinemic patients lack anti-suppressor cells.

Significance: These studies clearly demonstrate that immunoregulatory abnormalities are associated with patients with relatively mild Crohn's disease. It is possible that such cells may be a feature of normal feedback suppressor mechanisms which are set into motion as a result of the inflammation and cell activation occurring in the GI tract (see discussion of immunoregulatory defects in primary biliary cirrhosis below). More interesting is the possibility that the covert suppressor cell has a primary role in the causation of the disease. This could come about if excessive suppressor cell activity is generated in response to normal mucosal immune stimulation and this excessive suppressor activity results in immune suppression at mucosal sites which leads to initiation or perpetuation of the inflammation characteristic of Crohn's disease. Alternatively it is possible that appropriate feedback suppressor activity is being generated but that there is failure of suppressor cells to home to mucosal sites and exert suppression at mucosal sites where immune stimulation by lumenal antigens is constantly present.

Proposed Course: Several lines of research are opened by the research outlined above. 1) Most importantly we plan to study cells obtained from disease bowel segments of CD patients as well as diseased control patients to determine if increased or decreased suppressor T cell activity is present at this site and how such activity correlates with the presence or absence of circulating suppressor T cells. In this way we hope to gain insight into the question posed above as to whether or nor excessive or deficient suppressor T cell activity at mucosal sites can explain the disease on a primary basis. 2) We plan to widen our study of covert suppressor T cells to include patients with related diseases, patients under various drug regimens and relatives of patients with Crohn's disease. In the latter regard, if covert suppressor T cell activity is found in related but unaffected individuals then this immunoregulatory defect may be regarded as a primary rather than secondary abnormality. 3) Finally, it has been reported that patients with CD, usually those with very active disease, have Concanavalin A-induceable suppressor T cell activity as well as reduced autologous mixed lymphocyte reactions. At first sight this finding seems to contradict our own finding of increased suppressor T cell activity. However, it is possible that the reduced activity is found because the cells in the peripheral circulation are capable of responding to Con A to become auppressor T cells have already been activated as a result of the disease process as part of a normal or abnormal suppressor T cell feedback circuit (see discussion of suppressor

T cell circuits and the autologous MLR below). We therefore have initiated a study designed to correlate the presence of covert and excess suppressor T cell activity observed in PWM-induced Ig synthetic systems with the presence of reduced Con A-induceable suppressor T cell activity observed in mitogen-induced proliferative systems. We predict that the two will occur together for reasons alluded to above.

In summary, these studies of Crohn's disease conceivably offer considerable insight into normal regulatory function by allowing us to study the occurrence of anti- or contra-suppressor cell activity as well as allowing us to study the role of feedback suppression in chronic inflammatory states.

STUDIES ON IMMUNOLOGIC DEFECTS IN PRIMARY BILIARY CIRRHOSIS

Background: Primary biliary cirrhosis (PBC) is a progressive inflammatory disease of the liver which leads to intrahepatic cholestasis, cirrhosis and, eventually, hepatic failure. It occurs mainly in females and is not susceptible to treatment with corticosteroids.

Certain features of primary biliary cirrhosis suggest that immunological factors play an important role in the pathogenesis of the disease. These include pathologic changes in the liver such as lymphocytic infiltration of bile ducts, granulomas, lymphoid aggregates, and piecemeal necrosis of hepatocytes, the presence of elevated titers of non-organ specific auto-antibodies such as anti-mitochondrial antibody, the occurrence of high levels of circulating immune complexes, increased complement catabolism, and the frequent association of PBC with other diseases which are suspected of having an immunologic basis.

In previous studies of this condition we found that when cell cultures containing syngeneic mixtures of purified B cells and T cells obtained from PBC patients were set up, the T cells failed to manifest suppressor cell activity on PWM-stimulated Ig synthesis normally manifest at high T cell/B cell ratios. However, such suppressor activity was seen in cultures containing purified patient T cells and allogeneic normal B cells. This finding suggested to us that patients are not capable of generating suppressor cells as a result of autologous interactions but are capable of generating such cells as a result of allogeneic interactions. In studies of PBC reported last year we investigated this possibility by studying the ability of patients' T cells to mount proliferative responses when exposed to autologous and allogeneic non-T cells. In these studies we found that the patients had a marked deficiency in the ability to generate an autologous mixed lymphocyte reaction (MLR), but have a normal ability to generate an allogeneic MLR.

Because we feel that autologous cell interactions are very important in the cellular abnormality in PBC we have now begun to investigate the cellular basis of the autologous MLR. As background information, it should be mentioned that the autologous MLR is a well recognized immune reaction involving responding T cells and stimulating non-T cells (macro-

phages, B cells and null cells). The reaction shows specificity and memory and appears to involve surface antigens controlled by the histo-compatibility locus (D-region locus).

In the present series of investigation we determined the functional capabilities of the cells activated in the AMLR. In these studies, purified T cells were exposed to B cells or macrophages to elicit an AMLR in a primary culture of autoreactive T cells. The T cells thus obtained were then re-isolated from the primary culture and added to a secondary culture composed of B cells alone plus PWM to measure helper effects of the autoreactive T cells or to a secondary culture composed of B cells and fresh irradiated T cells plus PWM to measure suppressor effects of the autoreactive T cells. In these systems B cells alone would not be expected to be induced to synthesize Ig by PWM without addition of helper T cells whereas B cells and irradiated T cells would produce optimal amounts of Ig and would, therefore, be susceptible to suppressor effects of activated T cells.

It was found that autoreactive T cells (pre-stimulated by either B cells or macrophages) were less able to help B cells produce Ig than fresh T cells or T cells cultured alone (without stimulating B cells or macrophages). In contrast autoreactive T cells (pre-stimulated by either B cells or macrophages) were able to significantly suppress Ig synthesis by B and T cells indicator cultures. This suppressor capability was x-ray and steroid sensitive and was not manifest when autoreactive was added three days after inhibition of culture, indicating that the suppression was not due to cytotoxicity. These results indicated that the predominant functional effect of activation of T cells in the autologou MLR is to suppress PWM-stimulated Ig synthesis. In a second series of studies it was observed that the proliferation of T cells in culture with autologous non-T cells (ie., in an autologous MLR) is increased by a factor of ten if the stimulating cell population is pre-activated. In studies exploring this finding it was found that both PWM-transformed B cells and EBV-transformed B cells were able to activate suppressor T cells, suggesting that the mechanism of activation is independent of the agent used to stimulate the lymphoid cells. In addition, PWM-stimulated cells acquire maximal ability to activate suppressor T cells only after three days of culture with mitogen, indicating that lymphoid cels have to express new antigens or else display old antigen differently in order to activate T cells in the autologous MLR. As in the studies reported above the auto-reactive T cells stimulated with autologous activated cells (lymphoblastic B cells) express suppressor capability but little helper capability.

Significance: These studies point to the conclusion that the autologous MLR results, at least in part, from surface antigens expressed on cells (B cells) after cell activation. In addition, they show that the main functional sonsequence of the autologous MLR is the activation of a suppressor T cell population. In the light of these findings it is reasonble to suggest that through the autologous MLR, cell activation leads to feedback suppressor induction and that in the autologous MLR

one has a polyclonal equivalent to feedback suppressor circuits previously described in antigen specific systems.

These new observations help explain the defect in PBC. We now interpret the inability to generate a autologous MLR in this condition as an inability to generate feedback suppressor cells. This, therefore, explains the suppressor T cell deficiency noted in our earlier studies. The defect in the capacity to generate autoreactive suppressor T cells observed in PBC can be generalized to other autoimmune states. In this regard the defect in PBC is probably also present in SLE and constitutes a general defect which, in part, accounts for the autoimmunity seen in these conditions. However, other disease factors must also exist which gives each disease its own unique character.

Proposed Course: The results obtained above suggest several additional studies of PBC and the autologous MLR. 1) Most importantly we must more directly relate the autologous MLR defect with defective suppressor T cell activity. For this reason, PBC T cells will be stimulated by autologous non-T cells and resultant autoreactive T cell population evaluated for suppressor activity. The latter will be accomplished by adding PBC autoreactive cells to indicator populations composed of B cells and irradiated T cells as indicated above. In this study, the capacity of autoreactive T cells to mediate Ig suppression will be compared to the capacity of alloreactive T cells to mediate suppression. In preliminary studies of this type we have found that PBC patients do indeed have a defect in their capacity to generate autoreactive suppressor T cells whereas their capacity to generate alloreactive suppressor T cells is intact. 2) The identity of cells proliferating in the autologous MLR will be determined using monoclonal antibodies of the OK-T series. In these studies, cells population will be selectively depleted of helper-inducer and suppressor/cytotoxic subsets. The resulting cell populations will then be studied for capacity to proliferate and mediate suppression following autoactivation. In preliminary studies of this kind it has been found that OK-T8 positive cells are required for expression of suppressor cell activity. Using this approach we hope to identify the specific cell population in PBC that is defective. 3) The nature of the antigen on stimulator cells capable of activating autoregulatory T cells will be studied. In other studies performed elsewhere it has already been determined that antigens controlled by histocompabilitity genes (D-region antigens or Ia antigens) are important in the autologous MLR. We plan to investigate if other cell surface antigens also play a role in this reaction. First to be considered is surface Ig since activation of B cells leads to increased in surface Ig density and as indicated above, activation of B cells augments the ability of this cell to induce T cell proliferation. 4) Finally, the cells responding in the autologous MLR will be characterized more completely. In these studies we plan to establish longterm cell lines of autoreactive cells with the use of T cell growth factors (IL-2) and to identify the surface phenotypes and functional characteristics of the cell line thus obtained. In these studies we plan to compare the characteristics of autoreactive cells with alloreactive cells and with antigen-reactive cells. In addition,

we plan to use each cell line to obtain monoclonal antibodies specific for autoreactive cells using hybridoma technology.

In conclusion, these studies should allow us to define, on a cellular level, the precise nature of a immunologic defect which accounts for autoimmunity in a representative autoimmune disease, primary biliary cirrhosis. These studies should, therefore, allow further studies of this defect on a molecular level.

STUDIES OF GLUTEN-SENSITIVE ENTEROPATHY:

Background: Studies directed toward understanding the pathogenesis and treatment of gluten-sensitive enteropathy (GSE) were continued. GSE is a disease of the GI tract associatd with malabsorption, villous flattening, lymphocyte infiltration of the lamina propria and an increased incidence of neoplasia. It is due to the toxic effect of wheat proteins (gliadin) on the gastrointestinal mucosa. In previous studies we observed that approximately 80% of GSE patients bear the HLA-B8 histocompatibility antigen. In addition, we have shown that circulating B cells, but not T cells of patients bear a surface antigen which is detected with antibodies found in mothers of patients with GSE. Furthermore in family studies we have found that this "B cell antigen" is inherited independently of the HLA antigens and may therefore be the gene product of a non-HLA-linked gene necessary for the occurrence of GSE.

Findings: For a variety of reasons it is possible that GSE is due to disordered immune response to gliadin protein. In this regard it is possible that GSE results from a genetically determined disorder of class-specific antigenspecific regulation of the immune responses. To substantiate this possibility we have developed a method of stimulating peripheral cells of patients with GSE in vitro with purified gliadin protein fractions. In this method the cells of patients are placed into culture with either a control antigen (influenze virus) or test antigen (gliadin fraction) and at the end of 7-14 days the culture supernatants are examined with a solid phase assay for antigliadin antibodies which utilized enzyme-linked anti-immunoglobulins. We are currently accumulating data on patient with this technique.

Prizes and Awards:

Distinguished Achievement Award, 1981;
American Gastroenterological Association

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-04003-25-MET

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies of Porphyrin Metabolism in the Tumor-Bearing Host and Porphyria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald P. Tschudy, M.D. Senior Investigator MET NCI

COOPERATING UNITS (if any)

Laboratory of Viral Carcinogenesis, NIAID
Laboratory of Parasitic Diseases

LAB/BRANCH
Metabolism Branch, DCBD, NCI

SECTION

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NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	1	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main objectives are to study the mechanisms controlling heme biosynthesis and biochemical aspcts of porphyrin metabolism in experimental porphyria, tumors and tumor bearing hosts.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To study mechanisms controlling heme biosynthesis and biochemical aspects of porphyrin metabolism in experimental porphyria, tumors and tumor bearing hosts.

Methods Employed: Quantitative enzyme determinations, chemical determination of porphyrins, porphyrin precursors and tissue heme; use of various tissue and cell preparations and isotope methods to study metabolic pathways; measurement of lymphocyte proliferation in response to mitogens and antigens; measurement of serum hemolytic antibody levels.

Major Findings:

Succinylacetone (4,6-dioxoheptanoic acid), an irreversible inhibitor of the second enzyme of the heme biosynthetic pathway, when administered by intraperitoneal osmotic minipump for two weeks, produced a 100% survival rate in rats that had received 10^6 Walker 256 carcinosarcoma cells (IP) and rats that had received 10^6 Novikoff hepatoma cells (IP). It had only slight anti-tumor activity against leukemia L1210 *in vivo*.

This compound prevented the allograft rejection reaction seen when the Walker 256 carcinosarcoma is grown as a solid tumor (sc) in outbred Sprague Dawley rats. The profound immunosuppressive activity of succinylacetone was further demonstrated in two other systems. It markedly inhibited the proliferation of human peripheral blood lymphocytes in response to both mitogens and antigens. Succinylacetone adminsitration to Fischer rats inhibited by 99% or more the rise of serum hemolytic antibody levels in response to intraperitoneal injection of sheep erythrocytes. The antiproliferative effect of succinylacetone in some tumor cells (murine erythroleukemia cells) is reversed by administration of hematin, but in others (leukemia L1210 and the Wlaker 26 carcinosarcoma) is unaffected by hematin, even though all these tumor cells can take up hematin. It is thought that this compound can exert an antiproliferative effect by two separate mechanisms, one rleated to inhibition of heme biosynthesis and the other a heme independent mechanism. Since hematin did not reverse the inhibition of human peripheral blood lymphocyte proliferation produced by succinylacetone, it is thought that the immunosuppressive effects of this compound involve a heme independent mechanism. The doses of succinylacetone which produced profound immunosuppressive activity in rats were not accompanied by any evidence of gross toxicity.

Studies of hematin and porphyrin uptake by malignant cells have shown kinetics characteristic of active facilitated transport. Two separate mechanisms have been shown to augment uptake of heme and porphyrin by melignant cells. The first involves a gradually increasing capacity (over 3-4 days) for hematin and porphyrin uptake by tumor cells grown in the presence of succinylacetone. In the second mechanism there is a rapidly developing (30 minutes) augmentation of hematin and porphyrin uptake produced by certain local anesthetics and dimethylsulfoxide. Phototoxicity of hematoporphyrin treated tumor cells is maximum at a wavelength of 503 nm and is augmented by a brief period of protreatment with certain local anesthetics.

Publications:

Tschudy, D.P., Ebert, P.S., Hess, R.A., Frykholm, B.C. and Weinbach, E.C.: The effect of heme depletion on growth, protein synthesis and respiration of murine erythroleukemia cells. Biochem. Pharmacol. 29:1825-1831, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-04002-12-MET

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Defects in Immunoregulatory Cell Interactions in Patients with Immune Dysfunction.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thomas A. Waldmann, M.D.	Branch Chief	MET	NCI
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	Thomas A. Fleisher, M.D.	Clinical Associate	MET	NCI
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	Warner Greene, M.D.	Expert	MET	NCI
	Takashi Uchiyama, M.D.	Visiting Fellow	MET	NCI
	Philip Leder, M.D.	Branch Chief	LMD	NICHD
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LAB/BRANCH

Metabolism Branch, DCBD, NCI

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	4	2

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were directed toward determining the steps involved in the differentiation of B cells into immunoglobulin synthesizing and secreting plasma cells. Special emphasis was laid on developing new techniques to study the role of helper T cells, macrophages, and suppressor cells in these immune regulatory processes and to define defects in these immunoregulatory cell interactions in patients with immune dysfunctions. Leukemias of prosuppressor, suppressor effector and helper T cells have been identified. Excessive numbers of suppressor T cells have been demonstrated in association with agammaglobulinemia selective IgA deficeincy, suppressor leukemias, infectious mononucelosis, and post transplantation immunodeficiency states, antigen nonspecific suppressor factors that inhibit B cell immunoglobulin synthesis have been identified in the supernatants of cultured human T cell lines and T-T cell hybridomas. Recombinant DNA technology has been applied to study the arrangement and rearrangement of immunoglobulin genes in lymphocytic leukemias and in lymphocyte cell lines. In these studies it was shown that human B-cells rearrange the genes in the expressed allele and may retain, rearrange, or delete the non-expressed allele. Unexpectedly the kappa gene was deleted in lamda expressing B cells.

Project Description:

Objectives: The objectives of the study were: to determine the sequential steps involved in the differentiation of stem cells into B cells and then into immunoglobulin synthesizing and secreting cells. Major efforts were directed toward defining the immunoglobulin gene rearrangements and deletions that occur as a stem cell matures into a pre B-cell and to determine the role of the regulatory network of suppressor T lymphocytes, helper T lymphocytes, and macrophages in the control of the maturation of B cells into plasma cells. Disorders of these suppressor and helper interactions have been defined in the primary immunodeficiency, allergic, autoimmune and malignant diseases of man. Human T cell leukemias were examined for surface phenotype, retained immunoregulatory function and for the capacity to undergo further maturation. Furthermore, studies were directed towared identifying, isolating and characterizing human factors that suppress immunoglobulin synthesis. Overall the studies are directed at defining disorders of host immunoregulation that lead to immunodeficiency states that have a high incidence of malignancy or that led to immunodeficiency and a high incidence of infection in patients who have malignancies of the T and B cell system. Finally studies were directed toward developing new techniques for the detection of circulating tumor-related antigens that could be used in cancer diagnosis or in monitoring the effectiveness of therapy.

Methods Employed: In vitro culture techniques of mitogen stimulated peripheral blood lymphocytes have been developed for the study of the terminal differentiation of B lymphocytes into immunoglobulin secreting cells. New techniques with B cells purified by immunoadsorbent columns and followed by removal of contaminating T cells by rosetting techniques have been used in co-culture studies to detect helper T cells in man. Co-culture studies have also been utilized for the detection of excessive numbers or reduced numbers of suppressor T lymphocytes. Lymphocytes depleted of adherent cells by a variety of techniques as well as new monocyte purification procedures have been used to assess monocyte helper and suppressor functions. A major accomplishment was the development of a method to study antigen specific antibody synthesis by human peripheral blood cells in vitro that were stimulated by antigen in the absence of polyclonal activtors. Hybridoma procedures have been used to produce monoclonal antibodies to maturation antigens of T cells and to study biological modifiers of the immune response produced by T cells. Recombinant DNA technology with ^{32}P labeled C DNA probes to the human constant kappa, lambda light chain and the heavy chain genes were used to study the gene rearrangements in B cell maturation and to analyze disorders in lymphocytic leukemias and immunoglobulin deficiency states.

Major Findings: A major effort of the Metabolism Branch over the past few years has been directed toward defining the events of cellular differentiation, cellular interaction and cellular biosynthesis involved in the specific circulating immune response. These studies have placed special emphasis on defining the defects of B cell maturation and of regulatory T cell and macrophage interaction with B lymphocytes and

plasma cells that occur in patients with primary immunodeficiency diseases associated with a high incidence of malignancy, in patients with autoimmune disorders as well as in patients with malignancies of the T or B lymphocyte systems. Overall these studies were directed at defining the factors in normal and abnormal states controlling the production of antibodies and the synthesis of the immunoglobulin molecules. Cells that ultimately produce antibodies undergo sequential maturation from stem cells in the marrow to B lymphocytes and then from B lymphocytes into immunoglobulin synthesizing plasma cells.

The earliest events of maturation of stem cells into B cells involve rearrangements of the genes coding for the appropriate light and heavy immunoglobulin chains. These events have been examined for human cells in collaboration with Dr. Philip Leder and Dr. Phillip Hieter. Human light chain genes exist as discontinuous gene segments of V (variable), J (joining) and C (constant) regions in the germline state. During maturation of stem cells into B-cells a rearrangement occurs in the expressed gene bringing a single V region in contiguity with a specific J region. ^{32}P labeled clones of human kappa ($C\kappa$) and lambda ($C\lambda$) constant region genes were used as probes to study gene rearrangements in lymphocytes from 9 chronic lymphocytic leukemia patients, 7 Epstein-Barr virus transformed and 3 spontaneous B-cell lines. Genomic DNA was extracted from these cells digested with the restriction endonucleases known to demonstrate both rearranged and germline alleles, and hybridized with the probes. $C\kappa$ exists as a single gene in man. The BamHI digests of all 8 kappa expressing B-cells demonstrated at least one rearranged kappa allele. Four displayed a remaining germline allele, while 2 had double rearrangements and 2 had deleted the other allele. There are thus three different patterns accounting for allelic exclusion. The excluded allel may be germ line deleted or rearranged presumably in an aberent fashion. At least five $C\lambda$ genes were shown to exist in each individual in at least 2 germline, patterns due to polymorphism of EcoRl restriction sites. EcoRl digests of all 11 lambda expressing B-cells showed a rearranged lambda allele (3 displayed double rearrangements) not seen in fibroblasts or T-cells from the same individual. When genes coding for the opposite isotype were examined Kappa expressing B cells retained their lambda genes in the germ line configuration. However, lambda expressing B cell deleted or rearranged all of their kappa consistant region genes. Such kappa gene deletions usually involved the J_{kappa} as well as C_{kappa} region but spared a V_{kappa} family. These observations observations suggested an ordered hierarchy with kappa gene activation preceeding lambda. In contrast to B cells, human T cell leukemias and lines almost always retained their immunoglobulin genes in the germ line configuration. In order to study the earliest events of immunoglobulin gene recombination we have examined eight human "non-T, non'B" leukemic lymphocytes representing immature stages of lymphoid differentiation. Fully seven of these eight populations demonstrated immunoglobulin gene rearrangements indicating that most cases of this leukemia are already committed to B-cell development at the immunoglobulin gene level. In addition, the majority of heavy and light chain gene rearrangements observed were not accompanied by detectable cytoplasmic immunoglobulin suggesting that

many of these rearrangements may be of an aberrant nature. Such a population of B-cell precursors, rich in recombinational errors of immunoglobulin gene joining, may indicate that the somatic assembly of immunoglobulin genes is remarkably prone to error. Furthermore, the patterns of immunoglobulin gene rearrangement within these leukemic pre B cells suggests a hierarchy of somatic rearrangements with mu genes preceding light chains and kappa light chain genes generally preceding lambda.

The maturation of B cells into antibody producing plasma cells is carefully regulated both positively and negatively by distinct subpopulations of lymphoid cells. Specifically many antigens and mitogens require the presence of both helper lymphocytes of thymic origin (helper T cells) and macrophages as well as the B cells to induce a full antibody response. More recently it has been recognized that a separate class of thymic derived cells, suppressor T cells, may act as negative regulators of B cell maturation inhibiting this process. We and others have shown that suppressor T cells emerge from the thumus as inactive prosuppressor cells that require an interaction with another T cell (termed a suppressor inducer or activator) in order to become a final effector of suppression. To study these events we have developed a series of techniques including techniques to study the terminal differentiation of B cells into immunoglobulin synthesizing and secreting cells, techniques to assess helper T cell function, and to detect both increased and decreased functional activities of suppressor effector T cells and their precursors and activators. Previously we used a single basic procedure for these determinations in which peripheral blood lymphocytes were cultured in vitro with pokeweed mitogen, a polyclonal activator, and the IgG, IgA, and IgM synthesized and secreted by these cells was quantitated using radioimmunoassay procedures. Pokeweed mitogen is a prototype of a thymic and macrophage dependent activator of B cells that also has the capacity to activate some prosuppressor cells to effectors of suppression.

A major new accomplishment over the past year has been the development of a culture and assay system for the sensitization of human peripheral blood mononuclear cells with a T cell dependent antigen, sheep erythrocytes, in the absence of nonspecific stimulatory agents and with the subsequent generation of macroscopic hemolytic plaques. We have shown that the antibody produced by the plaque forming cells generated in this culture system is specific for the sensitizing antigen, and that the plaques created are not false plaques since their formation is inhibited by cycloheximide. The success of this system can be attributed to several critical factors including large numbers of peripheral blood mononuclear cells ($5x10^6$/culture), a prolonged period of incubation (10-11 days), continuous rocking during the entire period of incubation, culturing in large (35mm) flat bottom culture dishes in the presence of human plasma, and the appropriate antigen concentration ($5x10^6$ SRBC/culture). Futhermore, the generation of macroscopic hemolytic plaques requires plaquing sensitized peripheral blood mononuclear cells in target cell monolayers fixed in an agarose matrix with an incubation period of two to three hours. We have further shown that the antigen specific response measured by this system

is dependent on adherent cells and T lymphocytes. At least one population of the helper T cells is sensitive to 2000R irradiation. This system is simple, sensitive, and should serve as an effective tool for the analysis of cellular interactions involved in the generation of human antigen specific PFC, the genetic control, the human immune response, and the pathophysiology of altered immunoregulation in disease.

In the past year we have made a significant breakthrough by developing a culture and assay system to assess in vitro antibody synthesis by human peripheral blood mononuclear cells stimulated by antigen without any polyclonal activator. This system shows antigen specificity (e.g. sheep red cells, TNP modified cells), macrophage and T cell dependence. At least one of the T cells required is radiosensitive. This system has been used to show that patients with ataxia-telangiectasia have a helper T cell defect as well as their defect in B cells.

Using the in vitro biosynthesis procedures with polyclonal activation we developed we have identified patients with disordered immunoglobulin synthesis due to a variety of mechanisms including disorders of intrinsic B cell activity, disorders of helper T cell function, disorders of the network of interacting T cells involved in immune suppression as well as disorders of monocyte function. Patients with hypogammaglobulinemia with primary B cell defects that we have defined include patients with x-linked agammaglobulinemia, patients with certain types of common variable immunodeficiency, with selective IgA deficiency or with the immunodeficiency characterized by elevated IgM levels and reduced IgG and IgA levels.

A second area of interest has been in helper T cell activity in animal models and in patients with disorders of immunity. In clinical studies deficiency of helper T cells was observed in patients with severe combined immunodeficiency disease with B cells and a patient with common variable hypogammaglobulinemia and in some patients with ataxia telangiectasia. An extreme defect in helper T cells was shown to be the cause of the hypogammaglobulinemia in the form of severe combined immunodeficiency characterized by an absence of circulating T cells but normal numbers of B cells. The B cells of these patients could not synthesize immunoglobulin molecules when cultured with pokeweed mitogen in vitro but synthesized immunoglobulin normally when normal T cells were added to the culture system. The primary defect in such patients might be a defect of T stem cells or to a failure of thymus function leading to a failure in the maturation of such stem cells into functional T cells. A similar pattern of hypogammaglobulinemia with normal B cell function has been identified in a patient with common variable hypogammaglobulinemia who has a T cell defect limited to the helper T cell series.

The technique for assessing helper T cell activity were applied to homogenous populations of T cells from patients with T cell leukemias. One of 20 acute T cell leukemias and seven of 12 patients with Sezary T cell leukemias retained the capacity to help normal B cells in pokeweed mitogen stimulated cultures. It is of interest that these patients had high IgA and IgE

levels and in 11 cases the Sezary cell leukemia has been associated with a circulating monoclonal immunoglobulin.

We are preparing an antisera to the idiotype of the heavy chains of these monoclonal immunoglobulins to determine if they react with the putative T cell receptors since it has been suggested that helper T cells for specific B cell clones share immunological determinants with the hypervariable region of the heavy chain of the specific antibody whose production is stimulated by the T cells.

Over the past year we have analyzed leukemic T cells with retained functions with monoclonal hybridoma antisera that define T cell subsets. Some of these antisera obtained commercially define T cell subsets with different functions (e.g. OKT 4, helper T cells, OKT 5, 8 suppressor cytotoxic T cells) whereas others define the state of T cell maturation (e.g. OKT 10 immature cells, OKT 3 mature T cells, Ia activation antigens on T cells). We have also used a monoclonal hybridoma anti-T cell antibody we have prepared ourselves that defines a 120,000 mol. wt. antigen termed Tac on the surface of activated T cells. Normal thymocytes and circulating T cells do not bear this antigen whereas T cells activated with mitogen, the mixed leukocyte reaction, or T cell growth factor do. All acute T cell leukemias bear the OKT 10 antigen but rarely bear the OKT 4 or 5 antigen, and in only 5% of cases the OK 3 maturation antigen. The seven Sezary leukemia cells studied in contrast do not bear OKT 10 but are all OK 3 and OKT 4 (e.g. mature helper phenotype) positive and are OK 5, 8 (e.g. suppressor phenotype) negative. They are also negative for Ia and Tac antigens. Thus the studies with monoclonal antibodies is in accord with our previous conclusions that Sezary cells are relatively mature T cells that are dedicated to helper interactions with B cells.

We have demonstrated excessive suppressor T cell activity in the neonate and in association with a variety of immunodeficiency states including infectious mononucleosis, one form of T cell leukemia, a subset of patients with common variable hypogammaglobulinemia, thymoma and hypogammaglobulinemia and some patients with selective IgA deficiency.

Studies on three patients with T cell leukemias with retained suppressor activity have been continued in order to address one of the most critical issues concerning the capacity of T cells to regulate human immunoglobulin synthesis, that is whether suppressor cell activity is generated by a single population of T cells acting alone of whether suppressor cell generation requires interaction between different populations of T cells neither of which has the capacity to affect suppression alone. The proof of such T-T interaction requires clear identification and separation of the two classes of cells involved. In human systems, this has been exceedingly difficult until now. We have taken advantage of an unusual patient with acute lymphoblastic leukemia and hypogammaglobulinemia prior to therapy to address this issue. In our studies we learned that the neoplastic T cells from this patient functioned as pro-suppressor cells. These cells were OKT 10 + but negative for the other antigens discussed above. We observed that the patient's leukemic T-cells produced no detectable immunoglobulin and depressed the immunoglobulin production

of co-cultured normal unseparated lymphocytes by 85-100% in the presence of pokeweed mitogen. However, suppression was observed only when cooperating normal T cells and pokeweed mitogen were present. Prior irradiation of either the leukemic cells or the cooperating normal cells nulified the suppressor effect. We, thus, were able to conclude that an interaction between at least two different T cell subsets is required for the generation of so-called suppressor-effector T cells in man. We extended these studies by analyzing whether normal T cells could secrete a soluble factor which could activate the leukemic cells under discussion to become suppressor effector cells. We learned that normal T cells when stimulated with pokeweed mitogen secrete a factor (or a series of factors) which may activate the leukemic cells to become direct suppressors without the further need of cooperating T cells in a pokeweed mitogen driven system of immunoglobulin production. In a Nocardia water-soluble mitogen-driven system of immunoglobulin production (known to be relatively T-independent), it was learned that both pokeweed mitogen and these leukemic cells were required for suppressor effects. These studies enabled us to construct a model for the induction of human suppressor effector cells. In this model, pro-suppressor T cells must interact with a different activating set of T cells before becoming fully functional suppressor T cells. To our knowledge, these studies represent the first proof of cooperative interaction between neoplastic and normal T cells in the generation of immunoregulatory effector T cells. Furthermore, we showed that there was an alteration of the surface phenotype of the leukemic cells following incubation in vitro with the T cell inducing factors that paralleled the changes in function. Following incubation the leukemic cells became OK3 (mature phenotype) OK8 (suppressor phenotype) and Tac (activated phenotype) positive.

Patients with common variable hypogammaglobulinemia represent a complex group of disorders with different fundamental defects. Many have intrinsic B cell defects, a few have a deficiency of helper T cells and a few have a circulating factor that inhibits B cell function. A subgroup of patients have excessive suppressor cell activity. Synthesis of immunoglobulins by normal cells was suppressed by over 50% when cocultured with equal numbers of peripheral blood lymphocytes from 44% of patients with common variable immunodeficiency and by over 80% when incubated with the lymphocytes of 18% of the patients. The T cells from the patients with suppressor cells were shown to function as suppressors. In some cases suppressor monocytes were also present. In some cases suppression was only observed when pokeweed mitogen was used as the polyclonal activator, whereas in other cases, the cells of the patient suppressed when any polyclonal activator was utilized. The suppressor cells in the former case were steroid and irradiation sensitive and appear to be prosuppressors that require pokeweed mitogen for their activation. The suppressor cells that suppress in non-pokeweed mitogen stimulated cultures and that are corticosteroid resistant appear to be in vivo activated suppressor effector cells. In the one patient studied extensively the target of the suppressor cell was shown to be the B cell rather than the helper T cell which is the target of most antigen specific suppressor systems. The most critical question concerning the role

of the suppressor T cells in the pathogenesis of hypogammaglobulinemia has been addressed by isolating the patients' B cells free of the patients T cells and determining their capacity to synthesize immunoglobulin molecules in vitro. In some but not all cases we demonstrated that patient B cells freed of suppressor T cells could synthesize immunoglobulin molecules supporting the view that the suppressor T cells played a pathogenic role in the hypogammaglobulinemia. The patients with excessive suppressor cell activity had excessive numbers of Ia positive OK8 positive T cells.

Purified human peripheral blood mononuclear cells stimulated with the mitogenic lectin Concanavalin A (Con A), continuous cultures of human T cells grown in interleukin II as well as human T-T cell hybridomas have been prepared that elaborate a variety of immunoregulatory molecules including suppressive factors. We find evidence for at least two diffeent suppressor activities in the supernatants of these cells one of which negatively modulates in vitro immunoglobulin biosynthesis while the other inhibits T cell proliferation. Immunoglobulin production was studied using a pokeweed mitogen driven reverse hemolytic plaque assay. The humoral suppressor factor produced 40-80% inhibition of polyclonal antibody synthesis. This factor was: 1) of molecular weight 30-45,000 daltons, 2) noncytotoxic, 3) present as early as 8 hours after exposure to Con A, 4) reversed by the monosaccharide L-rhamnose but not by a variety of other simple sugars including alpha-methyl-D-mannoside, 5) produced by macrophage depleted T cell populations but not by B cells, 6) found in the supernatants of long term human T cell cultures. The second suppressor factor produced 40-85% inhibition of in vitro lymphocyte proliferation in response to the mitogenic lectins phytohemagglutinin and Con A and the antigens streptokinase-streptodornase and tetanus toxoid. this factor was also of molecular weight 30-45,000 daltons and noncytotoxic. Its effect, however, is blocked by N-acetyl-D-glucosamine but not by L-rhamnose. In contrast to the humoral factor, the production of this factor required the presence of macrophages. We conclude that activated suppressor elaborate different soluble factors which independently modulate humoral and cellular immune reactions.

A modification of the immunoglobulin biosynthesis procedure has been developed to assess the ability of macrophages to act as one of the helper cells required for B cell maturation and to detect activation of monocytes into cells that inhibit immunoglobulin synthesis. Optimal immunoglobulin synthesis induced by pokeweed mitogen, staphage lysate and streptolysin O were dependent on the presence of macrophages. If excessive numbers of normal macrophages (i.e., over 50% of the cells in the culture system) profound suppression was observed. T cells were not involved in this suppression as participants or targets. The suppression does not act via cytotoxicity. Thus it appears that normal macrophages can be activated to inhibit B cell maturation and immunoglobulin synthesis. Patients with multiple myeloma have a disorder of the monocyte suppressor system with excessive numbers of activated suppressor monocytes that lead to the reduced polyclonal immunoglobulin synthesis observed with these patients. We have demonstrated a similar monocyte suppressor disorder associated with a monocyte produced suppressor factor in mice bearing transplantable myeloma tumors.

Significance to Biomedical Research: The development of techniques for the study the effect on helper and suppressor T cells on the maturation of B cells and their application to the study of patients with immunoglobulin disorders have been of great value in defining the critical stages of B cell maturation and of the network of immunoregulatory cells that control this maturation process. A series of new pathogenic mechanisms have been defined to explain the immunodefects in patients with immunodeficiency, autoimmunity, allergy and malignancy. Leukemias of immunoregulatory cells have been defined assisting the classification of leukemias. Furthermore, the studies of leukemias with recombinant DNA technlogy are providing insights into the ealiest events of B cell maturation and are binding the clasisificaiton of malignancies. In general, these studies are providing a scientific basis for developing rational strategies for the therapy of immunodeficiency and malignant diseases.

Proposed Course: Studies directed toward understanding the pathways and regulatory mechanisms controlling the sequential development of stem cells into B cells and then into synthesizing plasma cells. Special emphasis will also be placed on the genes and their rearrangement that control immunoglobulins and into the role played by the network of immunoregulatory T cells in controlling immune responses. The studies of the nature and mode of action of biological modifiers of the immune response secreted by cloned T cell lines and T-T cell hybridomas will be extended.

Honors and Awards:

G. Burroughs Mider Lecture, NIH
Meritorious Service Medal, DHHS
Second Annual Lecture in Allergy and Immunology, University of Puerto Rico Medical School
Plenary Lecturer of the Immunology Society of America

Publications:

Waldmann, T.A: Therapeutic manipulation: The application of new insights into the regulation of the immune response to be development of more rational therapeutic strategies for the prevention and therapy of human diseases with immunologic disorders. In Amos, B. (Ed.): Escape from Immune Surveillance: The Interface between Immune Mechanisms and Disease. New York, Academic Pres, Inc. pp. 303-309, 1981.

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SUMMARY REPORT
LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE
October 1, 1980 to September 30, 1981

The Laboratory of Pathology is responsible for the diagnostic services in Pathological Anatomy for the Clinical Center of the NIH and has research programs in various areas of experimental pathology. The Laboratory is divided into 10 sections:

A. Surgical Pathology and Postmortem Section
B. Cytopathology Section
C. Ultrastructural Pathology Section
D. Biochemical Pathology Section
E. Comparative Oncology Section
F. Viral Oncology and Molecular Pathology Section
G. Hematopathology Section
H. Pathological Technology Section
I. Office of the Chief
J. Image Processing Section

A. Surgical Pathology and Postmortem Section

Dr. Jose C. Costa together with Drs. Reichert and Barsky, the residents, staff pathologists from the Laboratory of Pathology plus Dr. A.W. Cheever (NIAID), Dr. W.C. Roberts (NHLBI), Dr. K.J. Stromberg (NCI), Dr. L.A. Liotta (NCI), and Dr. P.E. McKeever (NINCDS) were responsible for the surgical pathology and autopsy diagnostic services. 5,666 surgical specimens or biopsies were accessioned in the past year. 141 autopsies were accessioned. These specimens involved the preparation of 53,408 slides, including 36,023 H&E stained sections, 6,238 special stains and 605 frozen sections. Clinicopathological studies in pulmonary vasculitis, breast cancer, pancreatic carcinoma, esophageal carcinoma, recurrent hyperparathyroidism and soft tissue sarcoma, are in progress.

Many specimens of fresh human tissues, including the eyes which are regularly removed during a complete autopsy, are furnished to NIH scientists in various laboratories. A tissue procurement nurse works closely with the staff and helps in the distribution of specimens to scientists.

Conferences with individual clinicians and surgeons from the various Institutes are an important part of the care and study of patients in the Clinical Center. Regular gross specimen reviews of organs removed at autopsy are conducted by the staff and most surgical specimens are discussed at the weekly Surgical Pathology Conference conducted by Dr. Costa, residents and staff. In addition, the staff participates in other regular conferences including Pediatric Oncology Tumor Conference (Dr. Triche), Hematopathology and Lymphoma Staging Conferences (Dr. Jaffe and staff), a monthly Neuropathology Conference (Dr. Valsamis) and conferences on Sarcoma Staging, Surgical Morbidity and Mortality, Medicine-Radiation Therapy (Dr. Costa and staff). The staff and residents present selected autopsies at monthly clinicopathologic conferences (e.g. NCI - Dr. Costa, NHLBI - Dr. Roberts). Weekly

slide presentations by the residents using current surgical and autopsy cases are held as a part of their training program.

B. Cytopathology Section

This section provides diagnostic services in cytology (both exfoliative and fine needle aspiration) and medical genetics. The chromosomal analysis includes conventional and special banding techniques (C-, G-, and R-bands) for the examination of individual chromosome pairs. During the year, approximately 3600 cytology specimens were accessioned. These represent over 8,400 smears examined and reported. A little less than 1/4 were cervical and vaginal specimens; 3/4 were other types of specimens (including ascitic, pleural, gastric, spinal fluids, bronchial specimens (both brushing and washing), sputum and fine needle aspirations.

In addition to the diagnostic reports, the staff of this section collaborates closely with the clinical and surgical staff in various research projects. For example, 1) CSF evaluation of leukemic patients to assess the therapeutic effects reflecting the degree of relapse or remission; 2) chemotherapy effects (especially cytoxan) on the GU tract as seen in urine specimens; 3) chemotherapeutic and radiation effects on the exfoliative cells (sputum, fluids, vaginal smears, etc.); 4) maturation index in precocious babies. Cytological methods are also used in support of collaborative research with other scientists in NIH.

Dr. Chu also serves on the staff of Tutorial on Clinical Cytology sponsored by IAC, ASC, and AARM. She gives lectures and workshops on Fine Needle Aspiration.

C. Ultrastructural Pathology Section

This section provides diagnostic electron microscopy services for a diverse group of Clinical Center physicians, including NCI, NIAMDD, NHLBI, NIAID, and NINCDS, as well as submitted cases from outside physicians. This past year 254 cases were accessioned; over 175 were processed and diagnosed. The service is housed in a separate module and is essentially self-contained, which provides a convenient reference point for all diagnostic EM cases, including facilities to examine the cases as well as all records, tissues, etc. This facility also provides diagnostic training and clinical research opportunities for residents and fellows. Dr. Hung Chiang, a visiting fellow from Taiwan, has been learning diagnostic EM while investigating light and electron microscopic immunoperoxidase techniques for identification of collagen types and skeletal muscle proteins in human soft tissue tumors. Dr. Ming Xia, a visiting pathologist from the People's Republic of China, has been learning diagnostic EM and undertaking an autoradiographic study of the distribution of radioactive isotopes in normal and tumor tissues in collaboration with nuclear medicine. It thus appears to be serving well its intended purpose as a diagnostic and teaching facility for clinically relevant electron microscopy.

Laboratory of Pathology, DCBD, NCI

Dr. Triche has continued a fruitful collaboration with Dr. Chaviva Isersky-Carter (and Dr. Henry Metzger) in NIAMDD. A study of the effect of IgE cross linking was completed this year, following completion of studies on the fate of monomeric IgE on rat basophil leukemia cells last year, which indicated IgE was slowly shed but never (within 72 hours) internalized unless crosslinked (by dimethyl suberimidate in this case). Further, this phenomenon is independent of histamine release, since dimerized IgE is internalized as well as larger oligomers yet fails to elicit exocytosis of histamine granules. The ultimate fate of these receptor-IgE complexes is under active investigation. Preliminary data indicate a novel mechanism for disposition, quite unlike the lysosomal destruction or receptor reshuttling recently reported in other related systems such as the insulin receptor, LDL receptor, or asialoglycoprotein receptor. This complex also appears to differ in its disposition from the IgG-Fc receptor complex. Recent work by Dr. David Segal has demonstrated the presence of an Fc γ receptor on these cells. Our studies have shown independent binding of immunoglobulin and disposition of immunoglobulin-receptor complexes by these cells; IgG-Fc γ complexes appear to be routed to the phagosome-lysosome compartment, while IgE-Fc$_\varepsilon$ complexes are not.

In a separate but related project, we have investigated the use of a rigorously purified monomeric IgE-ferritin conjugate as a label of cellular IgE disposition. Initial studies reveal a previously unappreciated non-uniformity of cell surface labelling as well as apparent internalization of some label. A detailed comparison of the biological disposition of radiolabelled IgE versus IgE-ferritin is thus under investigation. The initial results suggest that studies of the biologic fate of radiolabelled ligands versus ligand-ferritin conjugates may not be comparable, and that radiolabelled ligands may more nearly approximate, if not duplicate, the fate of normal, unlabelled ligands.

The IgE-ferritin conjugate has also been used to label membrane vesicles derived from basophils, in a multifaceted study of the IgE-Fc receptor complex. These studies, now complete, suggest that the Fc$_\varepsilon$ receptor is not a transmembrane protein, in that various techniques fail to detect an inner membrane facet expression of the receptor, in contrast to the readily detectable external expression of same.

Work on human tumor tissue and human tumor cells in vitro continues with Dr. Dickman and Dr. Liotta. Significant progress in the detection and qualitative analysis of tumor cell synthesized procollagen and collagen has been made. Ewing's sarcoma, for example, appears to synthesize a unique array of collagen types (I, III, and IV), not detected in any other tumor studied or reported. In contrast, a human fibrosarcoma which has been cloned synthesizes FN only in some clones; all clones and the uncloned tumor produce almost exclusively type III collagen. A variety of other tumors and their cultured progeny are also being studied, and preliminary results support a strong association between tissue of origin and collagen type synthesis - ie, osteosarcoma, of bone origin (type I collagen), synthesizes almost exclusively type I collagen. Another "fibrosarcoma" obtained elsewhere, though, synthesizes only type IV collagen. A detailed analysis of

the line by ultrastructure suggests it is in reality an adenocarcinoma, thus explaining the apparent anomalous type IV collagen synthesis.

Dr. Triche completed a chapter on Pediatric Tumors for the book "Cancer in the Young", edited by Dr. A. Levine of the Pediatric Oncology Branch, as well as a review of "round cell" tumors of childhood for Perspectives in Pediatric Pathology, edited by Harvey S. Rosenberg. The latter review was invited by Dr. Rosenberg after a presentation of similar material at the Pediatric Pathology Club interim meeting in St. Louis (10/80), invited by the meeting organizer, Dr. John Kissane. Dr. Dickman also presented his work on Ewing's sarcoma at the same meeting. Drs. Dickman and Triche are currently completing a chapter on the use of various newer techniques such as immunofluorescence/immunoperoxidase, biochemical extraction, immunoprecipitation, column chromatography and SDS-PAGE for diagnostic purposes in the detection and characterization of occult primary tumors presenting as metastatic disease (Cancer Invasion & Metastasis, ed. by L. Liotta and I. Hart, Martinus Nijhoff pub.; Hingham, Mass.). Additional work on the morphologic characterization of pediatric soft tissue tumors, presented at the 1981 IAP meetings by Dr. Dickman, is also being completed for publication.

Drs. Triche and Dickman participate fully in the surgical pathology service; they also present patient material in the weekly pediatric oncology tumor board.

D. Biochemical Pathology Section

The Biochemical Pathology Section is carrying on research on immunochemistry of complex carbohydrates. Current approaches include 1) determination of carbohydrate structures of glycoproteins and analysis of oligosaccharide mixtures by gas chromatography/mass spectrometry (GC/MS), 2) development of hybridoma antibodies against oligosaccharide haptens, and 3) studies on the origin, metabolism, and excretion of a urinary oligosaccharide [$(Glc)_4$] derived from glycogen.

1) Equipment and laboratory procedures for chemical analysis of complex carbohydrate structures have been set up and structural studies conducted by Dr. Nilsson and Ms. Cashel. Determination of the structures of oligosaccharides covalently bound to hCG and haptoglobin are nearly completed. Equipment to survey the composition of oligosaccharide mixtures released from cell membrane fractions by GC/MS has recently been installed. A chemical method for increasing the volatility of oligosaccharides that contain amino sugars developed by Dr. Nilsson will enhance these studies.

2) A hybridoma antibody suitable for use in a radioimmunoassay to analyze for $(Glc)_4$ as a free oligosaccharide is being sought to replace the rabbit anti-$(Glc)_4$ antibody now in use in clinical studies. Hybridomas have been produced by fusing mouse SP 2/0 myeloma cells with lymph node or spleen cells of mice immunized with a synthetic $(Glc)_4$-KLH glycoconjugate. Clones are selected for production of antibody that binds $(Glc)_4$-BSA and/or a derivatized ^{125}I-labelled

oligosaccharide hapten. Drs. Schroer and Lundblad are carrying out these studies with the assistance of Mr. Fernandez.

3) Studies on urinary excretion of $(Glc)_4$ in patients with soft tissue sarcomas are being conducted by Dr. Zopf, Dr. Ugorski and M.S. Levinson in collaboration with Dr. Pizzo of Pediatric Oncology. Preliminary data suggests that elevated excretion of $(Glc)_4$ in patients with rhabdomyosarcoma, Ewing's sarcoma, ALL, and some other tumors might be useful indicators of tumor regression or recurrence during therapy. Studies on the kinetics of formation of $(Glc)_4$ from glycogen during treatment with amylase are being conducted by Dr. Ugorski.

E. Comparative Oncology Section

The investigative work of this section is conducted by a member of the Senior Pathology Staff, Dr. Dawe; a Post-doctoral Fellow (NSF), Dr. Fisher; a Biologist GS-12, Mr. Morgan; a Biologist GS-11, Mrs. Williams; a Biomedical Technologist GS-9, Mr. Summerour; and a Biomedical Technologist GS-7, Mrs. Brown.

During the past year, research has been centered on 3 areas as follows:

I. Developmental and Genetic Factors in the Mouse Polyoma Virus Tumor System.

a. Interactions between polyoma (Py)-transformed salivary epithelium (PTSE) and normal fetal mouse dermis have been studied as a part of the continuing examination of epithelial-mesenchymal interactions during and after Py transformation. The work has been completed and a publication will be prepared in the coming year. It was found that PTSE induced melanin synthesis when placed in contact with normal melanoblasts in fetal mouse dermis. Pigmented melanocytes transferred melanin granules to PTSE as if the PTSE were recognized as normal hair follicle epithelium. In a corollary experiment, normal salivary epithelium interacting with normal dermis was found able to form hair follicles complete with sebaceous glands and well-developed hair shafts. The observations show a close developmental relationship between salivary gland and pilosebaceous apparatus and suggest that this relationship has a bearing on the tropism of Py for salivary gland and hair follicles, among other Py-responsive organs. The findings also offer a rationale for the heretofore puzzling condition known as Fordyce's spots, in which sebaceous glands develop from accessory salivary glands of the oral mucosa, in elderly people.

b. Dr. Fisher's isozyme studies of Py-induced tumors of several epithelial types showed profiles for 30 enzyme systems to be constant for each organ of origin, but different from organ to organ except for the salivary and mammary glands, which had identical profiles. Spontaneous mammary and salivary gland tumors had unique isozyme profiles differing within the respective sets and differing also from polyoma tumors of corresponding organ of origin. In addition, polyoma tumors derived from salivary epithelium transformed by Py in vitro differed in their isozyme signatures from salivary

epithelial tumors induced in vivo. These results indicate that the phenotype of polyoma-transformed cells is largely dependent on the phenotype of the target cell at time of transformation. The phenotypes of the normal cells that become transformed in vivo are evidently very constant for each organ, hence the emerging tumor phenotypes are also constant for the respective organs concerned. In this respect, Py transformation resembles certain normal developmental phenomena.

c. Experiments in collaboration with Dr. Mark Israel investigate the relationship between Py genetic variants and Py organ tropism. Early incomplete results indicate that plaque-cloned virus strains show reproducible differences in relative frequencies of tumors induced in various organs, as well as in lethality for newborn mice. Dr. Israel plans to perform genetic analyses of the virus variants, in an attempt to correlate viral genetic structure with selectivity for particular organs and with lytic effects vs. transforming effects.

A review of the work of the polyoma project was presented by Dr. Dawe in November, 1980, in the Nakahara Lecture at the 11th International Cancer Symposium sponsored by the Princess Takamatsu Fund, in Tokyo.

II. The Identity of X Cells in "Tumors" of Certain Fishes.

X cell lesions (papillomas and pseudobranchial tumors) have been reported in the literature to involve fishes of 3 Orders (Pleuronectiformes, Gadiformes, and Perciformes) including about 40 species. For some 70 years these lesions have been considered neoplastic or preneoplastic. The enigmatic nature of X cells (previously thought to be neoplastic cells) was brought to attention in 1969. Dr. Dawe, in collaboration with Dr. A.J. Garvin, Dr. S.E. Fisher, Ms. Mary Habbersett, Mr. C.M. Poore, and Mr. J. Bagshaw, conducted an investigation of X cells to determine whether they are actually of host fish-cell origin. In imprint preparations in this study, X cells stained by Wright's, Feulgen, and a DNA-binding fluorescent dye (bisbenzimidazole), the features of the mitotic cycle of X cells were visualized in detail and found to be closely similar to those of hartmannellid amoebae. Mitoses of X cells differed markedly from mitoses of host fish cells. In multinucleated plasmodia-like forms, mitoses of the several nuclei in a given cell were synchronized. Isozyme studies revealed starch gel electrophoresis bands extractable from X cells but not from host fish cells, active in at least 4 to 6 enzyme systems. Flow fluorometric quantitation of DNA showed the X cell population to be sharply distinct from that of host fish cells, with X cell/fish cell DNA ratio of about 1:3. It was concluded that X cells are parasitic rather than neoplastic in nature.

A report of these findings was presented in Tokyo in November, 1980 and will be published in the Proceedings of the 11th International Cancer Symposium sponsored by the Princess Takamatsu Fund.

Laboratory of Pathology, DCBD, NCI

III. Studies of Myoepithelial Contractions in Organ Culture.

In previous work, Mr. Morgan demonstrated and characterized myoepithelial contractile activity in mouse salivary gland, using time-lapse cinematography. During the past year he has worked with lactating mouse mammary gland. Most of his effort was devoted to developing a satisfactory culture system, and he has recently succeeded in demonstrating contractions in glands taken 4 days postpartum. Contractions appear to be more vigorous in this system than in developing salivary gland. Expulsion of milk from mammary gland alveoli can be visualized. Further work is planned to determine contraction and relaxation times, synchronization features, and possibly hormonal influences.

F. Viral Oncology and Molecular Pathology Section

The investigative studies ongoing in this section are carried out under the direction of four principal investigators: Drs. Howley, Rabson, Costa, and Arya. These studies are primarily involved with animal viruses with an emphasis on the human viruses. The concerns are the mechanisms by which these viruses interact with cells susceptible to lytic productive infection and with cells which can undergo malignant transformation.

Dr. Arya's studies involve the biologic and virologic characterization of breast and prostate cancer cells. In his studies involving retroviruses in mouse mammary tumor cells, Dr. Arya has found that cells which initially synthesized only a type B retrovirus (MMTV) with passage continue to produce increasing amounts of type C retrovirus. This was accompanied by a marked increase in the synthesis of type C viral RNA but not by a decrease in the MMTV RNA. In addition, mouse interferon inhibited the production of both viruses equally but did not have an effect on the intracellular concentrations of the two viral RNAs. An analysis of the polyomavirus genomes in nude mouse mammary tumors has revealed multiple copies of the viral genome integrated as long tandem repeats within the host genome. In addition, in analyzing the sequence complexity and diversity of the messenger RNA in cultured human breast carcinoma cells and human prostate carcinoma cells, has revealed that approximately 80% of the sequences are held in common.

Dr. Costa, working with Dr. Thorgeirsson and Ms. Yee, has continued studies with the induction of Fc receptors in cells infected by human herpes viruses. They have shown that the Fc receptors increase with induction of the EBV lytic cycle in lymphoblastoid lines. In addition, with T.S. Tralka as well as Ms. Yee, Dr. Costa has been analyzing type A viral particles in a human chondrosarcoma which has been transplanted into nude mice.

Dr. P. Howley and Dr. F. Rentier-Delrue have continued their studies with the human polyomaviruses, particularly the human polyomavirus JC. In studies involving Drs. M.-F. Law, and C. Heilman from the Laboratory of Pathology, as well as Drs. Mark Israel and Ken Takemoto of NIAID, they have cloned the genome of the Mad-1 strain of JC into pBR322. Dr. Rentier-Delrue and Dr. Howley in collaboration with Dr. A. Lubiniecki have purified JC DNA directly from two human PML brains using the differential salt precipitation technique of Hirt.

The DNAs were quite homogeneous in size and they cloned them directly into pBR322 at the single Bam HI as well as single Eco RI sites. These DNAs were compared with the Mad-1 strain of DNA by restriction endonuclease analysis and only minor differences were noted between these various JCV DNAs. The region of variance was mapped to a small region of the genome, mapping to the late side of the origin of DNA replication. This region is a non-coding region in the corresponding areas of the SV40 and BK genomes and is believed to be a region involved in transcriptional regulation.

Drs. Howley, Law, Heilman, and Sarver have continued their studies with the human and bovine papillomaviruses. In collaboration with Drs. Lowy and Dvoretsky (Dermatology Branch, DCBD, NCI) they have shown that the BPV-1 and BPV-2 DNAs which they have cloned are capable of transforming susceptible rodent cells and have shown that a subgenomic transforming segment of the BPV-1 genome is capable of inducing this transformation. Dr. Law has established that the BPV viral DNA is present in the mouse cells transformed by either intact virus, the linear cloned BPV-1 DNA or the linear subgenomic transforming segment, is present exclusively in an unintegrated extrachromosomal state in these mouse cells. Dr. Heilman is currently analyzing the viral specific messenger RNAs that are present in these transformed mouse cells. Equivalent studies done with the human papillomaviruses have failed to demonstrate a transforming capability for these viruses.

Drs. Howley, Sarver, and Law in collaboration with Drs. Khoury and Gruss (Laboratory of Molecular Virology, NCI) have exploited the extrachromosomal nature of the BPV genome in transformed mouse cells and demonstrated the utility of this DNA as a cloning vector in eukaryotic cells. Specifically, they have linked the rat preproinsulin 1 gene to the transforming segment of the bovine papillomavirus genome and used it to transform susceptible mouse cells. Selecting for the malignant transformed phenotype, they have demonstrated that these cells faithfully transcribe the DNA which is maintained extrachromosomally and that authentic rat proinsulin is made within these cells. Current studies are under way further evaluating the utility of this viral vector system. In collaboration with Drs. Art Levinson and Peter Seeburg of Genetech, Inc. of South San Francisco, California, Dr. Howley and his coworkers are sequencing the entire 8,000 base pair genome of the BPV-1.

G. Hematopathology Section

Drs. Jaffe, Cossman, Crabtree, Kant, Neckers, and Simrell have major programs in diagnostic and experimental studies of neoplastic and non-neoplastic lesions of the lymphoreticular and hematopoietic system. Drs. Jaffe, Cossman, Crabtree, and Kant are Board Certified Pathologists who take responsibility for the diagnostic material on cases of malignant lymphoma admitted to the various clinical services in the Clinical Center. The above staff participate in two weekly conferences for the clinical staff of the Medicine Branch and Radiation Oncology Branch, DCT, NCI. These include a weekly Clinicopathologic Conference on current cases as well as a more formal weekly Staging Conference for malignant lymphomas. In addition, Drs. Jaffe and Cossman participate in the diagnostic services of the

Laboratory of Pathology, taking responsibility for both Surgical Pathology and Postmortem Pathology, and as such, supervise residents involved in these services. Drs. Jaffe and Cossman also receive several hundred cases submitted for diagnostic consultation from pathologists in the regional medical community, as well as throughout the United States.

The Hematopathology Section has an active research program in several areas. The section has continued its work on the immunologic, cytochemical, biochemical, and functional aspects of human malignant lymphoma. This program has been exceptionally active with over 125 fresh biopsy specimens analyzed during the past year from patients with malignant lymphoma admitted to the Clinical Center. Areas which are receiving particular emphasis include the clinicopathologic correlations of this immunologic data. A study on the correlation of immunologic phenotype with conventional morphology, showing that immunologic phenotype cannot be predicted by morphologic criteria alone, is in press. Currently being analyzed in collaboration with the Medicine Branch, DCT, is the clinical importance of immunologic phenotype in patients with diffuse aggressive non-Hodgkin's lymphoma. This data is based on a large series of patients studied prior to therapy at the Clinical Center. In preparation is an extensive clinicopathologic study of peripheral T cell lymphomas. The Hematopathology Section of the Laboratory of Pathology has collected the most extensive series of peripheral T cell lymphomas in the United States, and over 25 cases of this rare clinicopathologic entity are being reported in detail in terms of their clinical, pathologic, and immunologic aspects.

The peripheral T cell lymphomas are associated with several unique clinicopathologic syndromes. Recently published is a study demonstrating production of osteoclast activating factor by the neoplastic cells leading to profound hypercalcemia. These lymphomas are also associated with a syndrome which simulates malignant histiocytosis both clinically and pathologically. Dr. Simrell has pursued this clinicopathologic observation experimentally and has shown that certain normal and neoplastic T lymphocytes elaborate a factor or factors which induce IgG Fc receptors, phagocytosis and adherence of human macrophages. The induction of phagocytosis may be mediated via an increase in Fc receptors, since phagocytosis of IgG-coated particles is always increased whereas that of inert particles usually is not. The effect on macrophage adherence seems to be mediated by a separate factor.

In the near future these studies on malignant lymphomas will be extended to the molecular level with an analysis of the presence or absence of immunoglobulin gene rearrangement. This study will be performed in collaboration with Drs. Stanley Korsmeyer, Thomas Waldman, and Philip Leder.

Dr. Jeffrey Cossman in the past few months has initiated a new investigative area in the Hematopathology Section with the acquisition of a Fluorescence Activated Cell Sorter by the section. He is employing a variety of monoclonal antibodies to study both normal human lymphocytes as well as neoplastic cells derived from malignant lymphomas. The extensive material available to the Hematopathology Section provides a unique resource to characterize these monoclonal antibodies as to their specificity and rela-

tionship to discrete stages of differentiation or activation in the lymphoid system. In addition, new monoclonal antibodies against human B cells are being developed by Dr. Cossman. These monoclonal antibodies have relevance in the future for both diagnosis and treatment of human malignant lymphoma.

In collaboration with Drs. Cossman and Jaffe, Dr. Neckers, an Expert in the Hematopathology Section, is pursuing the significance of merocyanine 540, a unique extrinsic fluorescent probe, which may serve as a marker for transformed lymphocytes. Dr. Neckers is also pursuing his studies on the monoaminergic receptors on lymphocytes.

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Drs. Jaffe and Cossman's contributions in the field of neoplastic hematopathology have been recognized in the past year by the numerous invitations both have received to give lectures or seminars at scientific meetings in the United States and abroad. Dr. Jaffe continues to serve as a faculty member for the Tutorial on Neoplastic Hematopathology organized by the University of Chicago under the scientific direction of Dr. Henry Rappaport. Dr. Jaffe also participated in the annual meeting of the International Academy of Pathology: 1) chairing a scientific session, 2) participating as a panel member on a Specialty Conference in Hematopathology, and 3) serving as a faculty member for a two day course in Immunopathology. Dr. Cossman participated in the International Academy of Pathology as a faculty member chairing a course on the Immunologic Aspects of Malignant Lymphoma.

Dr. Jaffe has been invited to present a paper on the Importance of Immunologic Markers for the Pathologist at an International Conference on Malignant Lymphomas to be held in Lugano, Switzerland. Dr. Jaffe was recently appointed co-chairman of the Expert Panel on Cytochemistry for the International Committee for Standardization of Hematology. She continues to serve on the Editorial Board of the American Journal of Clinical Pathology. Both Drs. Jaffe and Cossman have been invited to participate in many educational and scientific sessions.

Another major research effort is being conducted by Drs. Crabtree and Kant, and has been remarkably productive over the past year. Drs. Crabtree and Kant recently developed a system by which 15 to 38-fold increases in the levels of translatable fibrinogen mRNA could be induced in the livers of Sprague-Dawley rats by inducing defibrination with Malayan pit viper venom. Using this phenomenon in a creative and innovative way, they have been able to obtain cDNA clones for the α, β, and γ chains of fibrinogen. They have shown that the genes are not independent but are coordinately regulated. This unique approach has relevance not only for this work but could be used to obtain recombinant plasmids for other families of coordinated regulated genes. Four major publications related to this work are either submitted for publication or in preparation.

In addition, Dr. Crabtree has been invited to present papers at two major meetings representing the highest level of work in the field of Molecular Biology, the UCLA-ICN symposium on Molecular Biology and the First Annual Congress on Recombinant DNA Research. In addition, in recognition of the unique nature of their accomplishments, they have received numerous invita-

tions to speak on their work at major scientific institutions including Roswell Park Memorial Hospital, University of Texas Medical School at Galveston, the Rockefeller University in New York, and Medical College of Virginia. Dr. Crabtree has been asked to present his work at a upcoming symposium at the New York Academy of Sciences. In recognition of his broad knowledge and experience in this field, he has been invited to present an Overview of the Regulation of Gene Expression at New Perspectives in Oncology for the 80's, a meeting to be held in early 1982.

In recognition of their professional accomplishments the staff of the Hematopathology Section have been invited to author chapters for several already published or upcoming books on topics including the pathology of pediatric and adult lymphomas, the use of histochemistry in the diagnosis of malignant lymphoma, the identification of membrane receptors in tissue sections, and analysis of steroid receptors in human benign and malignant lymphoid cells. Dr. Jaffe is currently involved in editing a book entitled "Surgical Pathology of Lymph Nodes and Related Organs".

H. Pathological Technology Section

The technology staff, under Mrs. Barbara Coolidge, is located in Bldg. 37. The section also has a working laboratory located at Ft. Detrick in Frederick, Maryland. They provided all types of histological services and staining procedures for scientists in DCBD and other divisions of NCI. During FY 81 they processed more than 18,000 pieces of animal tissue, cut and stained more than 47,000 H & E slides and prepared over 6,000 special stained slides. They also prepared over 13,000 unstained for immunoperoxidase studies and other special techniques. All types of animal tissue were accepted in their laboratory and the more routine work is sent to private contractors for sectioning and staining. There was a total of 76 NCI investigators using the services of the section. In addition, a number of specimens was submitted for embedding and sectioning for electron microscopy. The quality and performance of the Pathological Technology staff continues to be outstanding and they represent a major technical resource for the research of scientists in the NCI. The section also prepares study sets for the NCI Tumor Registry which are used by scientists all over the world as well as NCI.

I. Office of the Chief (Dr. Stuart Bentley)

During the current reporting period, we have continued to investigate the role of interaction between stromal and hematopoietic cells in the microenvironmental support of hematopoiesis.

Further characterization studies have been carried out on marrow-derived adherent cells (MDAC). These cells have been shown to provide a microenvironment which supports hematopoiesis *in vitro*. Experiments in which MDAC were cultivated from T6 negative radiochimeras bearing T6 positive bone marrow transplants indicated that the MDAC population was exclusively non-

hematopoietic - and therefore stromal - in origin. These stromal derived monolayers supported hematopoiesis in continuous bone marrow culture.

We have previously shown that MDAC consist largely of collagen-producing cells. Biochemical studies indicated that fresh suspensions of marrow cells include a proportion of collagen-producing cells. Immunofluorescence staining of frozen sections of rat bone marrow demonstrated collagen synthesis by cells which were morphologically indistinguishable from reticulum cells.

Further biochemical studies were undertaken to quantitate and to characterize collagen synthesis by stromal cells in intact marrow and in MDAC culture. Fresh marrow cells produced collagen types I and III in a ratio of approximately 5:1, collagen synthesis comprising about 0.2% of total protein synthesis. During MDAC cultivation, there was an increase in the collagen synthesis fraction by a factor of approximately 20, indicating enrichment of the cultures with collagen-producing cells. However, the type I:type III ratio was maintained in culture, suggesting selection of a single cell type with a stable type I:type III collagen synthesis ratio.

Projects currently in progress include studies of the role of fibronectin in the stromal:hematopoietic interaction. Fibronectin synthesis by MDAC has been confirmed biochemically. Ultrastructural examination of continuous marrow cultures stained by the PAP technique for fibronectin indicates the presence of this protein in a minority of cell:cell interactions. This will be studied further.

Studies of proteoglycan synthesis by bone marrow stromal cells are planned, in collaboration with NIDR. It is further intended to study synthesis of connective tissue proteins by marrow stromal cells in Steel anemic (Sl/Sl-d) mice. This genetically-determined anemia is due to an abnormality of the hematopoietic microenvironment. Since the latter appears to be provided by the marrow connective tissue, it would be especially interesting to determine whether or not there is an associated connective tissue biochemical abnormality in this disorder.

J. Image Processing Section

The Image Processing Section has continued and extended the biological as well as the biomedical related computer science productivity of previous years. In-section and collaborative research continued to center around the following major areas: 1) computer-aided 2 dimensional polyacrylamide SDS gels, 2) nucleic acid morphology, 3) durable fiber (asbestos, fiber glass, etc.) effects on living cells, and 4) biologically oriented image processing research. A 3-year plan for the replacement and upgrading of the RTPPs with provision for color graphics was developed and its first stage implemented by procurement of a COMTAL dual user display (delivery due in 1st quarter of next fiscal year). The cell biology aspects of the section's research will be strengthened when Dr. Judith Greenberg, a seasoned cell biologist, joins our group in an expert capacity before the end of the reporting period. Promising efforts to resolve

the critical need for extensive and advanced statistical support in several of the above areas are in progress at this writing.

1. 2D Gel Analysis. A third major collaboration in this area was begun during the reporting period. GELLAB, our system for computer-aided 2D SDS PAGE analysis is being used by Dr. Thorgeirsson's group in the Laboratory of Chemical Pharmacology to reduce data derived from homogenates of several cell lines. It is clear that our remote location and the large volume of work they plan, make it impossible for them to rely on us for all of their gel analyses. We are continuing to support them at the maximum practical level until their own system is viable. In the latter connection, we are providing advice, algorithms and whatever other interim assistance reasonably possible to aid them in establishing their on-campus facility. The nature of the analytic problems and the effectiveness of the collaboration promise a continued strong and mutually beneficial collaborative relationship even after they obtain their own computer.

Our continued collaboration with Dr. Eric Lester, now in the department of Medicine, University of Chicago, was extremely fruitful during the reporting period. We were successful in exporting major components of GELLAB to the University of Chicago System-20, so that Dr. Lester can conduct his own analyses of gel images. GELLAB has been expanded to accept images from scanners other than our TV input system, and thus Dr. Lester and others with access to a System-20 can become even less dependent on day to day use of our system. The successful export involved adapting GELLAB to run under a TOPS-20 monitor, thus making the system even more available to the scientific community. At the same time our substantive collaboration continues with measurements being performed on both systems as independent checks and with the planning and execution of additional experiments involving the lymphocyte-phytohemagglutinin system for the characterization of proliferative lymphocytic diseases.

A somewhat different biological approach characterizes our third (and oldest) major collaboration. Here Dr. Carl Merril's group in NIMH are looking for diagnostic differences in protein patterns in various diseases such as Alzheimer's, Huntington's chorea, Lesch-Nyhananomaly, etc. Because of the temporal variations and constraints on controls imposed by clinical considerations, the statistical problems attendant upon the gel production and analystic processes is even greater. Confirmation of apparent successes must await further replications and critical statistical analyses.

Work in the conjoint area of durable fiber effects on macrophage-like cells and the use of 2D gels for the analysis thereof is noted below.

At this writing at least a dozen papers dealing with GELLAB and its applications are published or in press.

2. Nucleic Acid Morphology. Work for the past year has involved extensive studies of computer techniques for synthesizing nucleic acid secondary structure given molecular sequence information derived from restriction enzyme studies. This work has in part been carried out in a collaborative effort with Dr. Jacob Maizel of the Molecular Structure Section of the National Institutes of Child Health and Human Development. Recent evidence suggests a direct connection between nucleic acid secondary structure and gene expression and therefore understanding the mechanisms that are involved in the formation of energetically stable nucleic acid molecular structures becomes exceedingly important. This highly combinatoric problem has been approached from both interactive and non-interactive viewpoints. Base paired region generation rules were combined with energy rules (stabilizing energies of paired regions and destabilizing energies of loop structures) to produce algorithms that generated and manipulated graphic matrices (a visualization technique for potential regions) of several molecules on the RTPP. The gray scale capability of the RTPP permitted the depiction of all possible regions along with their stabilization energies. Interactive techniques allowed a user to choose and/or alter various regions thereby interactively building a particular configuration for the molecule. The molecular configuration along with its morphologic entities, i.e., bulge loops, hairpin loops, internal loops and multibranch loops are available for user study. These techniques demonstrated a new configuration of a beta globin messenger RNA that had a better stabilization energy than that published in the literature. Profile histograms of the graphic matrix were also used in an attempt to find highly stable regions.

Experiments with non-interactive approaches were also performed in the search for an efficient algorithm for predicting secondary structure. These included region table based algorithms as well as sequence growing dynamic programming style algorithms. A region table based algorithm that executed in exponential time was modified and improved to run in cubed time (as a function of the number of regions), thus allowing a program that ran in 3 months time to run in a few hours. An algorithm that makes use of some dynamic programming techniques that runs in cubed time (as a function of the length of the sequence) is now also being studied and appears to be the best at this juncture.

A graphics molecular display program was also developed which permits the predicted molecular configurations derived from the above described programs, to be displayed either on a graphics display terminal or on a plotter. These computer generated molecular drawings then become quite close structurally to those molecular images seen in the electron microscope. Our goal is the eventual merger of some of the analytic techniques for producing secondary structure maps (developed previously) with the synthetic techniques described above, so that one may help the other, thus producing a tool that will help in understanding the rules that goven nucleic acid molecular morphology. Some of these results have recently been presented in a paper at the Medical Informatics conference in Strasbourg, France and will soon be appearing in a book entitled Computing in Biological Science.

3. Durable Fiber (Asbestos Etc.) Effects on Cells. A firm statistical basis for the bioassay of durable fiber effects has now been established. This is largely the result of a cordial and effective collaboration with Dr. Barry Margolin of NIEHS. Essentially the log linear dependence of cell number, our simplistic but useful criterion for cytoxicity, as a function of time has been established for both amosite and for aluminum oxide (serving as a prototype for the class of non-asbestos durable fibers). Detailed regression analysis has shown the reliability and utility of the cytotoxic effect and its correlation with fiber geometry and with the late Mearl Stanton's pleural sarcoma results employing the same fibers. Simplified techniques for counting field definition, data recording etc., should render the assay fully exportable during the next fiscal year.

The establishment of the assay now permits us to devote some of our limited resources to examining the why of this unique phenomenon. The basic question is how information concerning fiber geometry is "detected" by living systems such as P388D1 macrophages or pleural (?) endothelial cells. A series of experiments involving fluorescent labeled macrophage antibodies have thus far produced no unequivocal data. With the arrival of Dr. Greenberg, vigorous pursuit of both measures of cell motility and metabolic inhibitors begin with a view to more precisely examine and define the nature of fiber-cell interactions.

We have continued our collaboration with Dr. Tuchman-Duplessis of the French Academy. We have established to his satisfaction, the reasonable certainty that French attapulgite, used extensively in their pharmaceutical industry should not produce asbestiform effects, at least of the mesothelioma type. A further collaborative effort with the U.S. Bureau of Mines is directed toward obtaining fibers of more uniform dimensions so that a series of definitive experiments, testing the Stanton Hypothesis once and for all may be done. At the same time our bioassay is providing medical data for their industrial hygiene programs.

Work on human derived macrophages has produced results paralleling that obtained with P388D1. Because of non-proliferation of these cells and other biological variations, assays involving human cells are not as precise as those employing our cell line, but the results are otherwise confirmatory of our other experience.

Work on the polypeptides obtained from P388D1 macrophages analyzed by 2D PAGE technics continues. It has been these experiments that have definitively established the extent and absolute need for some sophisticated statistical support in order to establish the parameters of quantitative spot differences. The problem is sufficiently large to be beyond the personnel resources of our collaborators at NIEHS. We are exploring several other possibilities at this writing.

4. Image Processing Research. A variety of support software and hardware projects, necessary for the biologic research programs noted above were undertaken and completed during the reporting period. Prominent among these was the final derivation and implementation of a pointwise shade correction algorithm

that treats otherwise uncorrectable inhomogeneities in illumination and/or detection fields.

A biologically oriented image processing utility package for the System-20 was specified and developed during the reporting period. It is of sufficient generality so that additional hardware additions, such as the Comtal display will not require extensive rewrites.

The ominous prospect of PDP8 obsolescence coupled with the increasing scientific pressures for color display capabilities forced the formulation of a 3-year plan for the Comtal and its various peripherals. Briefly, we will be able to progressively increase image processing capabilities on this machine as dictated by the development of biological problems, while maintaining current capabilities and thus continue to meet our current commitments.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00853-28 LP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Surgical Pathology and Postmortem Section

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.C. Costa	Chief, Surgical Pathology & Postmortem Section	LP	NCI
OTHER:	S.H. Barsky	Expert	LP	NCI
	C.M. Reichert	Expert	LP	NCI

(See next page for additional staff)

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology and Postmortem Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
20	20	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Surgical Pathology and Postmortem Section, together with the Cytopathology Section, Ultrastructural Pathology Section and Hematopathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all Institutes in those investigations which involve the use and study of human pathological material.

The staff is engaged in several projects involving clinicopathological correlation and pathologic characterization of disease studied at the Clinical Center. Immunocytochemical techniques have been applied to the characterization and study of tumors and other non-neoplastic diseases.

PHS-6040
(Rev. 2-81)

Project Description

Staff:

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+Dr. Dana M. Fowlkes
+Dr. Sue Ellen Martin
+Dr. Timothy J. O'Leary
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*Dr. William C. Roberts, Pathology Branch, NHLBI
*Dr. Kurt J. Stromberg, Division of Cancer Cause and Prevention, NCI
*Dr. Marius Valsamis, Associate Professor of Pathology, New York Medical College, Center for Aging and Chronic Diseases

The objectives of the Surgical Pathology and Postmortem Section are: (1) to provide diagnostic services in pathologic anatomy; (2) to carry out independent research; (3) to conduct a residency training program in pathologic anatomy; and (4) to collaborate with National Institutes of Health investigators in research involving use and study of human material.

1. The service functions of the section during the past year included:

(a) 141 (130 "in house", 11 submitted) autopsy examinations. The residents perform nearly all of the postmortem dissections under supervision of the staff. A gross review is held weekly by the staff and residents. The residents review the microscopic slides of each autopsy with one of the staff before completing the autopsy protocol.

The staff assists the residents in preparing for the numerous clinical conferences in which the section participates.

+These physicians are full time Residents in the Laboratory of Pathology
*These Associate Pathologists spend part time in the activities of the Surgical Pathology and Postmortem Section

(b) 5,666 specimens were accessioned in the Surgical Pathology and Post-mortem Section. These specimens are initially examined and described by a resident and their reports are checked by the staff. Associate pathologists from other laboratories or sections are frequently consulted about problems in diagnosis in surgical pathology and/or assume responsibility for handling certain tissue removed for specific research projects.

(c) Histological preparation and special procedures: These functions are carried out under the direction of M.C. Bowling and R. Howard and a complete listing of specimens processed is given in the accompanying table.

(d) Photographic services of the department: A photographic record is made of the large amount of the pathological material which is handled and studied in the department. The photography is done by the staff and residents with the technical assistance of Mr. A. Calhoun and Mr. J. Banks who also maintain the equipment and carry out much of the photographic processing. Gross photographs and photomicrographs of the pathologic material are provided to the clinical staff on request, and are used extensively for conferences, seminars and many are prepared for publication.

A large proportion of the photomicrography is done under the direction of Mr. Ralph Isenburg, who provides professional assistance and facilities for the entire staff of the Laboratory of Pathology.

2. Independent research. The staff is engaged in the following projects:

a) Evaluation of the role of pathological classification and grading in the treatment of soft tissue sarcomas (Costa).

b) Pathological characterization of angiocentric lymphoproliferative disease involving the lung (Tsokos, Jaffe, Costa).

c) Characterization of the pathological picture in patients presenting with midline granuloma syndrome (Tsokos, Costa).

d) Characterization and study of ovarian neoplasms in patients with family history of ovarian disease (Kalavar, Costa).

2. Independent research. The staff is engaged in the following projects:

a) Dr. Cheryl Reichert is establishing a laboratory of immunocytochemistry.

b) Dr. Sanford Barsky is investigating the mechanisms of metastatis (under the direction of Dr. Lance Liotta).

c) Evaluation of the role of pathological classification and grading in the treatment of soft tissue sarcomas (Costa).

d) Pathological characterization of lymphomas with erythrophagocytosis (Tsokos, Jaffe, Costa).

e) Characterization and study of ovarian neoplasms in patients with family history of ovarian disease (Martin, Costa).

f) Study of tumors with neuroendocrine component (Barsky, Costa).

g) Study of pancreatic cell hyperplasia using computerized image analysis techniques (Lipkin, O'Leary, Costa).

h) Transplantation in nude mice of human tumors (Costa).

i) Participation in an international panel for the classification of nasopharyngeal carcinoma (Costa).

j) Study of DEN induced neuro-epithelial bodies in the hamster upper respiratory tract and lung.

3. Collaborative research.

a) Pathological study of the kidney in diabetic mice (Tsokos, Rabson).

b) Histochemical study of the distribution of creatine phosphokinase isozymes in human tissues including tumors (Zweig, Tsokos, Howard, Costa).

c) Pathological study of carcinoma of the esophagus (Glatstein, Martin).

d) Pathological characterization of excisional biopsies in breast carcinoma (Lichter, DeMoss, Costa).

e) Study of thyroid nodules (Barsky, Robbins).

f) Pathological features of testicular tumors (Barsky, Ozols, Javadpour).

g) Morphology of myositis ossificans (Zasloff, Barsky).

h) Study of peripheral nerve binding proteins (Reichert).

i) Study of cis platinum neuropathy (Reichert).

j) Study of pheochromocytomas (Linnoila, Keiser).

Ms. T. d'Angelo, a Tissue Procurement Nurse under the supervision of Dr. Costa, has provided 130 research specimens to the different laboratories at NIH (between 1-20-81 and 5-7-81). Twelve requests are being processed in order to obtain tissues from autopsies.

4. Conferences. The staff takes part in the following inter-departmental meetings:

Pediatric Oncology Tumor Board (weekly)
Sarcoma Staging Conference (weekly)
Surgical Morbidity and Mortality Conference (monthly)
Medicine-Radiation Conference (weekly)
Surgical Pathology Conference (weekly)
Lymphoma Staging Conference (weekly)
Testicular Tumor Staging Conference (weekly)

In addition, the staff has participated in 4 Clinical Center wide Clinico-pathological Conferences with outside invited discussants.

5. Data retrieval system. In conjunction with DCRT (Vincent, Dunham, Pratt) a program has been set up for storing and retrieving the surgical pathology and autopsy material. A program has also been devised to automatically encode all the diagnoses. The language used is a modification of SNOP and a dictionary is being generated for retrieval purposes. All the diagnosis in file in the Laboratory of Pathology since 1953 are now being re-formulated in order to make them suitable for automatic encoding. This is being done under the direction of Dr. Donald Henson. The cases from 1953 up to 1963 have been reformulated. A data file has been created that includes 1979 to 1981.

Proposed Course: (a) Continue to provide the services described. (b) Increase the interaction with the clinical branches in the design and evaluation of protocols. (c) Improve the opportunities for the resident staff to participate in teaching, conferences, and seminars, and provide elective periods to be spent accomplishing research projects with the senior staff. (d) Implement data retrieval programs.

Publications

1. Azar, H.A., Hansen, C.T., and Costa, J.C.: N:NIH(s)II-nu/nu mice with combined immunodeficiency: A new model for human tumor heterotransplantation. J. Natl. Cancer Inst. 65: 421-430, 1980.

2. Gelmann, E.P., Costa, J.C., and Myers, C.E.: Anaplastic sarcoma arising in a benign chondroblastoma: Case report and literature review. Orthoped. Survey 3: 379-385, 1980.

3. Reichert, C.M., Rosenberg, S.A., Webber, B.L., and Costa, J.C.: Malignant melanoma search for occult lymph node metastases. Hum. Pathol. 12: No. 5, May 1981.

4. Thorgeirsson, U.P., Costa, J.C., and Marx, S.J.: The parathyroid glands in familial hypocalciuric hypercalcemia. Hum. Pathol. 12: No. 3, March 1981.

5. Costa, J.C., Howley, P.M., Bowling, M.C., Howard, R., and Bauer, W.C.: Presence of human papilloma viral antigens in juvenile multiple laryngeal papilloma. Am. J. Clin. Path. 75: No. 2, February 1981.

6. Tsokos, M.G., Fauci, A.S., and Costa, J.C.: Idiopathic midline destructive disease (IMDD). A subgroup of patients with the "midline granuloma" syndrome. Am. J. Clin. Pathol., in press.

7. Martin, S.E., Dwyer, A., Kissane, J.M., and Costa, J.C.: Small cell osteosarcoma. Cancer, in press.

8. Webber, B.L., Heise, H., Neifeld, J.P., and Costa, J.C.: Risk of subsequent contralateral breast carcinomas in a population of patients with in situ breast carcinoma. Cancer, in press.

9. Costa, J.C., Webber, B.L., Muenz, L., O'Conor, G.T., Tabane, F., Belhassen, S., Mourali, N., and Levine, P.H.: Histopathological features of rapidly progressing breast carcinoma in Tunisia: A study of 158 consecutive cases. Int. J. Cancer, in press.

10. Costa, J.C.: The histopathological diagnosis of nasopharyngeal carcinoma. Cancer Oncol. Res., in press.

11. Hoofnagle, J.H., Dusheiko, G.M., Schafer, D.F., Micetich, K.C., Young, R.C., and Costa, J.C.: Reactivation of chronic hepatitis B virus infection after cancer chemotherapy. N. Engl. J. Med., in press.

12. Lanzer, W.L., Liotta, L.A., Yee, C.L., Azar, H.A., and Costa, J.C.: Synthesis of pro-collagen type II by a xenotransplanted human chondroblastic osteosarcoma. Am. J. Pathol., in press.

13. Yee, C.L., Costa, J.C., Hamilton, T., Klein, G., and Rabson, A.S.: Fc receptor activity in lymphoma lines is altered during Epstein-Barr virus production. Int. J. Cancer, in press.

14. Rabson, A.S., and Costa, J.C.: Effects of viruses on cells. Chapter in Medical Microbiology: Principles and Concepts., Publ. Addison-Wedley, in press.

15. Costa, J.C.: Nasopharyngeal cancer update - XII International Symposium of Dusseldorf. Hum. Pathol., in press.

SURGICAL PATHOLOGY AND POSTMORTEM SECTION

April 1980 through March 1981

SURGICAL PATHOLOGY	
Specimens accessioned	5,666
Blocks of tissue cut	15,116
Blocks of tissue recut	952
Slides made	53,408
Slides stained H&E	36,023
Slides stained special	6,238
Frozen sections	605
POSTMORTEM PATHOLOGY	
Autopsies performed	130
Autopsies submitted	11
Blocks of tissue cut	5,968
Blocks of tissue recut	701
Slides made	8,026
Slides stained H&E	6,102
Slides stained special	1,223
COMBINED TOTAL	
Total blocks cut	22,730
Total slides made	61,681
Total H&E	41,128
Total special stains	7,461

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00852-28 LP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Exfoliative Cytology Applied to Human Diagnostic Problems and Research Problems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.W. Chu	Chief, Cytopathology Section Medical Officer (Path.)	LP	NCI
OTHER:	P.R. DelVecchio	Super. Res. Biologist	LP	NCI
	T.A. Wood	Res. Biologist (Cytol)	LP	NCI
	L. Galito	Res. Biologist (Cytol)	LP	NCI
	A.M. Honig	Res. Biologist (Cytol)	LP	NCI
	K. Nakahara	Res. Biologist (Genetics)	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9	5	4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Provides complete diagnostic service in exfoliative cytology, medical cytogenetics, and fine needle aspiration cytology. In addition, collaborates in various clinical research projects utilizing special techniques including special staining and tissue culture techniques.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: 1. To provide the staff of the Clinical Center with an accurate and complete exfoliative and aspiration cytology service as well as diagnostic cytogenetics (karyotyping).

2. To collaborate in various clinical research projects evaluating cancer therapy, the hormonal status of the cancer patients, the course and natural history of the cancer lesions and the anatomical and physiological changes in the human body associated with various pathologic conditions.

3. Development of better diagnostic techniques.

The specific objectives include:

A) Cytology

1. Continued improvement in cytologic techniques in establishing definitive diagnosis.
2. Improvement in fine needle aspiration cytologic technique.
3. Establishing cytologic characteristics of cell types seen only in fine needle aspirations.
4. Setting up special stain techniques which may be used for general cytology or special research problems.
5. Maturation index in precocious babies.

B) Diagnostic Cytogenetics

1. Improvement in short term culture system to assure of successful harvesting of in vitro cells.
2. Improvement in staining techniques.
3. Establishing cytologic characteristics of cell types seen only in fine needle aspirations.
4. Improvement in special chromosomal analytical techniques such as bandings by various methods.

Methods Employed: 1. Special procedures in addition to the standard Papanicolaou stain are:

A) Millipore filtration of all fluids
B) Special stains:

1. Feulgan reaction for sex chromatin bodies.
2. Acid fast, Pneumocystis, Methenamine silver, Brown Brenn for various organisms and fungi.
3. PAS, Wright Giemsa, iron stain, melanin stain, congo-red, oil-red-O, mucicarmine for various specific reactions.

2. Short-term cultures of peripheral blood, bone marrow cells and tumor cells found in body fluids are the standard diagnostic methods.

In addition, special techniques involving trypsin digestion followed by special stains such as G-banding, Q-banding, and C-banding, as well as fluorescence staining whenever indicated.

Biopsy materials of skin, gonads, tumors are cultivated by tissue culture technique and cells harvested for chromosomal analysis.

Major Findings: Major contributions of the Cytopathology Section:

1. Providing prompt services and early diagnosis in cytological materials.
2. Providing cytologic evaluation and estimated ranges of either relapse or remission in the continual management of meningeal leukemia.
3. Providing cytological evaluation of therapeutic effects on cells seen in urinary specimens, vaginal-cervical smears, sputum and effusions.
4. Providing chromosomal analysis for clinical studies as well as in the establishment of definitive malignancies.
5. Providing cytologic methods to research projects specifically, fine needle aspiration in thyroid nodules.

Significance to Biomedical Research and the Program of the Institute:

1. The diagnostic value of exfoliative cytology in the clinical management of various disease states is an established fact.

2. Aspiration cytologic techniques are useful in establishing diagnosis on metastatic diseases and/or recurrent malignancies. In such instances, the fine needle aspiration technique eliminates the more involved incisional biopsy and therefore is more economical to the clinicians as well as to the patients.

3. Chromosomal analysis is a way of establishing definitive diagnoses in selected patients with endocrine disorders, congenital defects and also in some malignancies.

Proposed Course: 1. Continue to provide a complete cytodiagnostic service for the entire staff of the Clinical Center in various research projects.

2. Continual improvement and development of established as well as new techniques in cytologic and cytogenetic fields.

Activities: 1. Presented a series of lectures on Fine Needle Aspiration Cytology, China, September 21-October 4, 1979.

2. Lectures and Workshop on Fine Needle Aspiration of Thyroid Lesions. Tutorial on Clinical Cytology, Chicago, Illinois, May 9-15, 1980.

3. Lectures on Chemotherapeutic Effects, Cairo, Egypt, October, 1980.

Publications

1. Yron, I., Wood, T.A., Spiess, P.J., and Rosenberg, S.: In vitro growth of murine T-cells. J. Immunol. 12: 238-245, 1980.

2. Aboul-Enein, M.I., El-Bolkainy, M.N., Chu, E.W., Ahmed, T.H.E., Ibraheim, A.S., and Tawfik, H.N.: Evaluation of some field methods for the diagnosis of Schistosoma haematobium. Med. J. Cairo University 48: 335-341, 1980.

3. El-Bolkainy, M.N., Chu, E.W., Ghoneim, M.A., Shoukry, I., Raafat, M., and Ibrahim, A.S.: Screening of rural Egyptian population for the detection of bilharzial bladder cancer. Med. J. Cairo Univ. (Supplement, Dec. 1980).

4. El-Bolkainy, M.N., and Chu, E.W. (Eds.): Detection of Bladder Cancer Associated with Schistosomiasis. Al-Ahram Press, Cairo, Egypt, in press.

5. El-Bolkainy, M.N., Chu, E.W., Ghoneim, M.A., and Ibrahim, A.S.: Cytologic detection of bladder carcinoma in a rural Egyptian population infested with schistosomiasis. Acta Cytol., in press.

6. Higashi, G.I., El-Asfahani, El-Bolkainy, M.N., Chu, E.W., and Raafat, M.: Assessment of immediate and delayed hypersensitivity skin tests with Schistosoma mansoni adult worm antigen in a rural Egyptian population. J. Trop. Geograph. Med., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00518-03 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Fate of IgE Bound to Mast Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. Isersky	Senior Investigator	A&R	NIAMDD
	T.J. Triche	Chief, Ultrastructural Pathology Section (Surgeon)	LP	NCI
OTHER:	S.J. Mims	Biologist	LP	NCI
	J. Rivera	Biologist	A&R	NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Ultrastructural Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12	6	6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

IgE bound to the surface of mast cells and/or basophils is responsible for the immediate hypersensitivity reaction. This response once established, persists for prolonged periods of time. We have recently shown that the mechanism is not due to internalization (J. Immunol. 122: 1926-1936, 1979). Cross linking of the IgE by allergen (or other means) is normally necessary to elicit cell degranulation, which results in histamine release. We wish to determine if analogous, chemically induced cross linking affects the fate of IgE compared to monomeric IgE. The possible effect of oligomerized IgE binding to the recently described IgG Fc of basophils is also being investigated.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To study the effect of IgE cross linking on its fate after cell surface binding.

Methods Employed: 1. Radiolabelled IgE binding assays. 2. Electron microscopic autoradiography. 3. Statistical analysis using Salpeter's technique. 4. (computer assisted) digital analysis. 5. IgE ferritin labelling.

Major Findings: 1. Oligomerization of IgE with a chemical cross linker (dimethyl suberimidate) and subsequent binding of defined oligomers ($n \geq 2,3$, etc.) to cultured basophil leukemia cells results in time dependent internalization of these oligomers; suberimidate treated monomeric IgE, like untreated IgE, is not. 2. This internalization is temporally independent of the histamine release or exocytosis induced by these oligomers ($n \geq 3$). 3. Internalization and exocytosis are independent events, since dimerized IgE is internalized but does not elicit exocytosis. 4. Although IgE normally binds only to its receptor, oligomerized IgE displays an increased affinity for the Fcε receptor; oligomerized IgE also binds to the Fcε receptor. Despite this, the observed internalization of oligomerized IgE is not due to Fcε binding, since saturation of this receptor with IgG has no effect on IgE internalization and minimal effect on IgE binding.

Significance to Biomedical Research and the Program of the Institute: These findings will help clarify the mechanism by which hypersensitivity reactions, mediated by cell bound IgE reactive with specific allergens, can be provoked after prolonged periods of time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00520-03 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Surface Disposition of Cell Bound IgE with Time

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section (Surgeon)	LP	NCI
	C. Isersky	Senior Investigator	A&R	NIAMDD
OTHER:	S.J. Mims	Biologist	LP	NCI
	J. Rivera	Biologist	A&R	NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Ultrastructural Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antigenic responsiveness to allergens is imparted to mast cells and basophils by specific membrane binding of allergen binding IgE. Other cells have been shown to bind ligands non-randomly, especially to microvilli (dePetris, Nature 272: 66-68, 1978). Further, cell bound IgE has been shown to survive for prolonged periods of time on the cell surface (Isersky, Rivera, Mims, and Triche, J. Immunol. 122: 1926-1936, 1979). This study attempts to determine whether the prolonged survival is related to cellular re-distribution away from sites of active endocytosis; i.e., onto microvilli.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To determine the mechanism whereby mast cells and basophils retain cell bound IgE for prolonged periods of time.

Methods Employed: 1. Radiolabelled IgE binding studies. 2. Electron microscopic autoradiography. 3. Statistical analysis using Salpeter's technique.

Major Findings: Preliminary manual analysis indicates no preferential binding to microvilli when values are corrected for actual membrane surface area; uncorrected values suggest binding to microvilli only because of their greater membrane area. Despite this, the greater membrane area accounted for by these structures does result in greater IgE binding to microvilli.

A more detailed, computer-assisted analysis of these findings is underway. Initial results demonstrate a nearly two-fold greater density of IgE receptors on microvilli than cell surface using this more sensitive and reliable technique. A more comprehensive investigation is underway.

The time course of this binding is also being investigated.

Significance to Biomedical Research and the Program of the Institute: Understanding of the mechanism by which individuals retain immediate hypersensitivity to various haptens such as pollen and insect venom for exceedingly prolonged periods of time is hampered by an incomplete understanding of the cellular mechanisms by which IgE is retained on the cells mediating this response. This study is designed to clarify the mechanisms involved.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00530-02 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of Bile from Cases of Gallbladder Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D.E. Henson Medical Director LP NCI

COOPERATING UNITS (if any)

Dr. Roger Soloway, University of Pennsylvania

LAB/BRANCH
Laboratory of Pathology

SECTION
Ultrastructural Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Determine the composition of bile and gallstones from patients with carcinoma of the gallbladder as well as from certain racial and ethnic groups, e.g. American Indians, who are known to have a high incidence of gallbladder cancer.

Results indicate that Indians with gallbladder cancer have more glycolithicholis acid in bile than controls without cancer and that these patients do not completely sulfate the cholic acids. Reasons for incomplete sulfation are not presently known, but will be investigated.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Determine if there are differences in the composition of bile or stones from patients with cancer of the gallbladder as opposed to patients without cancer and determine reasons for the incomplete sulfation.

Methods Employed: Analytical chemistry methods, such as gas chromatography, thin layer chromatography, and column chromatography.

Significance to Biomedical Research and the Program of the Institute: Cancer of the gallbladder is common in some ethnic groups such as American Indians. Investigation of this disease in a defined population group could tell us about effects of diet and other factors on bile composition which ultimately would have a bearing on preventive measures. If the initial findings that patients with cancer do not sulfate completely prove to result from a defect in liver enzyme activity, then this will be the first demonstration of a metabolic defect in humans associated with cancer.

Proposed Course: Continue to analyze bile from patients concentrating on types of bile acids and mechanisms of sulfation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00545-03 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Collagen Synthesis by Human Tumors In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section (Surgeon)	LP	NCI
	P.S. Dickman	IPA Fellow	LP	NCI
OTHER:	L.A. Liotta	Surgeon	LPP	NCI
	G.R. Martin	Chief, Laboratory of Developmental Biology and Anomalies	LDBA	NIDR

COOPERATING UNITS (if any)

National Institute of Dental Research

LAB/BRANCH
Laboratory of Pathology

SECTION
Ultrastructural Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	6	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The type and amount of collagen synthesized by tumor cells in vitro appears to be altered from that of cultured normal cell counterparts (Stern et al., Cancer Research, Feb., 1980). We wish to broaden these observations to a variety of human sarcomas, and determine whether differentiation in vitro induced by differentiating agents will cause a reversion to more normal patterns of collagen synthesis, and whether these patterns might allow more precise categorization of the tumor's origins. In addition, the analysis will include a search for any new or atypical collagens unique to these tumors. Such collagens, if identified, might be valuable in tumor diagnosis.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: We wish to study collagen synthesis by normal and tumor cells in vitro, in cultures established by us from patients with known disease.

Methods Employed: These lines will be carefully evaluated for tumor cell growth, using all available techniques (chromosome analysis, density dependent growth, tumor production in nude mice, etc.). Collagen synthesis will be evaluated by ion exchange chromatography, slab gel electrophoresis, and autoradiography of radiolabelled cells.

Major Findings: 1. Tumor cells appear to produce greater amounts of type III procollagen than type I procollagen. 2. The effect of differentiating agents such as DMSO and HMBA on the ratio of type III/type I collagen synthesis is unclear, contrary to previous reports. 3. Total collagen synthesis, and the conversion of procollagen to collagen, must be quantitated before any meaningful comparison can be made. 4. Four Ewing's sarcoma cell lines have been investigated and have shown a unique collagen synthetic profile. These cells produce types I, III, and IV collagen, as demonstrated by immunofluorescence and gel electrophoresis techniques. This pattern has not been observed in any other tumors or tumor cell lines studied or reported. 5. Preliminary data from osteosarcoma, chondrosarcoma, fibrosarcoma, and carcinoma cultures and, in some cases, tumors, indicated characteristic collagen type synthesis - types I, II, III, and IV, respectively.

Significance to Biomedical Research and the Program of the Institute: In vitro correlates of malignancy are generally imprecise and unreliable. A reliable indicator of tumor growth as opposed to fibroblast overgrowth, in culture, is needed if meaningful study and comparison of tumor versus normal cells in culture is to proceed. Other protein candidates, such as fibronectin (or LETS), are not reliable. It is hoped that quantitative and qualitative analysis of collagen synthesis may provide a reliable index of tumor vs. normal cell biology.

Proposed Course: Ancillary techniques, such as quantitation of total collagen synthesis and quantitation of collagen by type as well as procollagen by type, utilizing immunoprecipitation techniques and eventually employing defined monoclonal type specific antisera, are being developed.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00592-07 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

A Morphologic Investigation of Virus Induced Membrane Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section (Surgeon)	LP	NCI
OTHER:	J.C. Costa	Chief, Surgical Pathology Section (Expert)	LP	NCI
	A.S. Rabson	Acting Chief, Laboratory of Pathology	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Ultrastructural Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	2	3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Herpes simplex virus infected cells develop a receptor capable of binding the Fc portion of IgG at the same time viral membrane antigens appear; it is not known whether this Fc receptor is a host cell or viral protein, though the latter is more likely. Studies utilizing hemocyanin-tagged IgG to label the Fc receptor and ferritin-tagged anti-HSV antigens on virally infected cells will determine whether the Fc receptor co-labels with antibodies to HSV antigens.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To demonstrate whether the herpes-induced Fc receptor is a viral protein or host cell protein modified by viral infection.

Methods Employed: 1. High resolution transmission (TEM) and scanning electron microscopy (SEM). 2. Immunolabelling with markers for TEM and SEM. 3. Freeze etch electron microscopy.

Significance to Biomedical Research and the Program of the Institute: The herpes-induced Fc receptor is of unknown function in vivo, but its ability to bind IgG suggests immune protective function for latently-infected cells expressing viral antigens on their surface. If this is so, the Fc receptor must occur over those areas of viral antigen to mask them. This study will demonstrate the spatial relationship of the two. If there is no relationship, the expression of an Fc receptor would be of doubtful survival value to the virus. If there is a relationship, convincing evidence for a novel biological survival mechanism will have been demonstrated.

Major Findings: 1. HSV-infected cells will bind Hcy-labelled IgG and ferritin-labelled anti-HSV antibodies, proving the approach is feasible. 2. Double label studies examined by TEM have so far been too sparsely labelled to detect significant co-labelling. 3. High resolution SEM, which might allow detection of the complexes, was unsuccessful. 4. Freeze etching of sandwiches of cultured cells at various stages of infection is underway.

Proposed Course: Freeze etching of virally infected cells, with and without immune complex labels, is underway. Initial technical difficulties are being circumvented and preliminary results are promising.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00594-05 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Pathology of Family Cancer Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.E. Henson	Medical Director	LP	NCI
OTHER:	J.J. Mulvihill	Head, Clinical Genetics Section	CEB	NCI
	J.F. Fraumeni, Jr.	Chief, Environmental Epidemiology Branch	EEB	NCI
	W.A. Blattner	Clinical Investigator	EEB	NCI
	M.H. Greene	Clinical Investigator	EEB	NCI

COOPERATING UNITS (if any)

Epidemiology Branch, NCI

LAB/BRANCH
Laboratory of Pathology

SECTION
Ultrastructural Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	5	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Review histopathology material from families with high incidence of cancer. These families are being followed by the Epidemiology Branch. Material is reviewed to confirm diagnosis and determine unusual aspects of the histology of the cancer in view of the family history.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Review and confirm the histological diagnoses on families that have a high incidence of cancer and which are being followed by the Epidemiology Branch.

Methods Employed: Light microscopy, special histochemistry stains.

Significance to Biomedical Research and the Program of the Institute: Confirm diagnoses of cancer and apply uniform terminology and classification to the lesions so that patterns may be found.

Proposed Course: Continue to review surgical pathology or autopsy material submitted from referring hospitals and report results to the Epidemiology Branch.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00508-04 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immune Response of CBA/N Mice to Oligosaccharides Coupled to Protein Carriers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Surgeon	LP	NCI
	K. Stein	Senior Staff Fellow	DBP	BOB, FDA
OTHER:	W.E. Paul	Chief	LI	NIAID

COOPERATING UNITS (if any)

Aftab Ahmed, Merck Institute, Rahway, New Jersey

LAB/BRANCH
Laboratory of Pathology

SECTION
Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

CBA/N mice are an inbred strain of animals that exhibit an X-linked deficiency in immune responsiveness to certain carbohydrate antigens including dextrans. Isomaltodextrins derived by partial enzymatic or acid hydrolysis of dextran were coupled as haptens to the protein carrier keyhole-limpet hemocyanin and were used as immunogens. These glycoconjugates were used to study formation of antibodies that bind dextran in normal adult and neonatal mice and in mice with the CBA/N defect. Of particular interest are studies of the size requirements for an oligosaccharide hapten to elicit a cross-reactive antibody response to the native polysaccharide and the ontogeny of the response to the polysaccharide following immunization with a glycoconjugate.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To investigate immune responsiveness of CBA/N and normal mice to isomaltodextrin oligosaccharide determinants coupled to a protein carrier.

Methods Employed: Isomaltodextrins are prepared by partial enzymatic or acid hydrolysis of Leuconostoc mensenteroides strain 512. Oligosaccharides are derivatized with β-(p-aminophenyl) ethylamine and coupled to KLH after activation to the isothiocyanate. Immune responses are monitored by isoelectric focusing of mouse sera or hemolytic plaque assays of spleen cells, and radioimmunoassays for various IgG subclasses.

Major Findings: Early studies of the anti-Dex response in mice with the CBA/N defect following immunization with IM6-KLH demonstrated that defective mice could make an anti-Dex response but that it was lower in magnitude than that of control animals. This suggested that the subset of B lymphocytes missing in the defective animals, the Lyb5$^+$ cells, might be important for dextran responses even when they are stimulated by a glycoconjugate. Previous studies by others had demonstrated that this B cell subset was required for the response to dextran immunization. To test this possibility, two types of experiments were performed: 1) neonatal mice which lack Lyb5$^+$ cells were immunized at various times after birth with IM6-KLH and the anti-dextran antibodies were measured and 2) a secondary adoptive transfer experiment was performed in normal adult mice in which IMG-KLH primed B cells, with or without anti-Lyb5$^+$ and complement treatment, were transferred to irradiated recipients and challenged with IM6-KLH and then the anti-Dex plaque-forming cell (PFC) response was measured.

The results of these experiments demonstrated that up to one week of age mice immunized with IM6-KLH do not make anti-Dex antibodies although they do produce anti-IM6 antibodies as detected with IM6-BSA. The anti-Dex cross reactive antibodies developed in parallel with the development of Lyb5$^+$ cells and reached a maximum at 3-4 weeks of age. This is considerably earlier than the response to dextran itself which does not reach a maximum until twelve weeks of age. These experiments reinforce the hypothesis that Lyb5$^+$ cells are required for the response to polysaccharides even when a glycoconjugate is used to stimulate that response. They also demonstrate, however, that once Lyb5$^+$ cells are present, glycoconjugates can stimulate an anti-polysaccharide response considerably earlier and of greater magnitude (100-1000 times higher titers) than polysaccharides themselves.

The adoptive transfer experiments were performed to directly assess the requirement for Lyb5$^+$ cells. In those experiments spleen cells from normal adult mice, primed to IM6-KLH, were treated in vitro with anti-Lyb5$^+$ and complement (performed in collaboration with Aftab Ahmed, Merck Institute, Rahway, N.J.) to remove the Lyb5$^+$ cells or were treated with a control antiserum and complement. These cells were transferred to irradiated recipients and boosted with IM6-KLH. Seven days later the spleens were removed from the recipients and the anti-Dex PFC response was measured. These experiments demonstrated that following removal of the Lyb5$^+$ cells, the primed cells were no longer capable of making an anti-Dex response. Taken

together these experiments clearly demonstrate that anti-polysaccharide responses depend on the presence of the $Lyb5^+$ cells whether the response is stimulated in a thymus independent on a thymus dependent fashion.

Significance to Biomedical Research and the Program of the Institute: The immune deficiency of CBA/N mice for response to polysaccharide antigens apparently can be circumvented when polysaccharide determinants are presented via a "T cell dependent" route. This finding opens a possible route to vaccination against bacterial organisms (e.g. pneumococcus) with surface polysaccharides that contain abundant repeating carbohydrate sequences but which may be poorly immunogenic in their native state. These studies also demonstrate that glycoconjugates stimulate 100-1000 times higher titers of antibody than the polysaccharide and significantly earlier in ontogeny than obtained using the polysaccharide. They suggest that glycoconjugates would be ideal for use as vaccines in infants that are unresponsive to polysaccharides.

Proposed Course: This project has been completed. Manuscripts describing the data are in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00510-03 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Glucose-containing Tetrasaccharide in Human Urine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Surgeon	LP	NCI
OTHER:	M. Ugorski	Visiting Fellow	LP	NCI
	R.E. Levinson	Biologist	LP	NCI
	P.A. Pizzo	Surgeon	PO	NCI

COOPERATING UNITS (if any)

Department of Clinical Chemistry, University of Lund, Lund, Sweden (Dr. Arne Lundblad)

LAB/BRANCH
Laboratory of Pathology

SECTION
Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies raised against a glucose-containing tetrasaccharide-Glcα1-6Glcα1-4Glcα1-4Glc-coupled to KLH have been used in a radioimmunoassay to measure urinary excretion of the oligosaccharide in urine of patients with glycogenoses, pregnant women, and pediatric patients with soft tissue sarcomas. Preliminary data suggest that the rate of urinary excretion of this tetrasaccharide may be a useful indicator of the tumor mass present in certain patients. The hypothesis that the oligosaccharide may originate as a product of digestion of glycogen by amylase is under investigation.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: 1) To determine the relationship between urinary excretion of a specific glucose-containing tetrasaccharide $(G)_4$ and altered states of glycogen metabolism. 2) To determine whether the urinary excretion rate of $(G)_4$ can be used as an indicator of tumor burden in patients with tumors that accumulate glycogen.

Methods Employed: A radioimmunoassay method was devised using rabbit antiserum prepared by immunization with a synthetic glycoconjugate containing $(G)_4$ coupled to KLH. Specificity of the assay was established by comparing relative activities of $(G)_4$ and several other glucose-containing oligosaccharides with related structures as inhibitors in the assay. The assay has greatest sensitivity and specificity for reduced $(G)_4$.

Major Findings: 1) Patients affected with types II and III glycogenoses excrete $(G)_4$ at rates 20 to 50 times normal. Unaffected parents, who are heterozygous for the recessive trait, excrete $(G)_4$ at approximately 10 times the normal rate. Some unaffected sibs of patients excrete $(G)_4$ at the same rate as their parents while others excrete normal amounts. 2) Urinary excretion of $(Glc)_4$ was elevated in 7 of 7 patients with Ewing's sarcoma, 9 of 12 with rhabdomyosarcoma, 3 of 4 with ALL, 1 of 4 with AML, and 2 of 7 with Burkitt's lymphoma. Of 21 hospitalized non-cancer patients, 3 who had chronic urinary tract infections had elevated urinary $(Glc)_4$ while the other 18 were in the normal range.

Significance to Biomedical Research and the Program of the Institute: The radioimmunoassay for $(G)_4$ in human urine has replaced previously used direct chemical analysis which required several days workup for each sample to be analyzed by gas chromatography/mass spectrometry.

Determination of urinary excretion of $(G)_4$ is easy, noninvasive, and inexpensive. $(G)_4$ excretion appears useful as an adjunct to other conventional methods for screening family populations suspected as carriers of recessive traits related to abnormal glycogen metabolism. Elevated excretion of $(G)_4$ in patients with tumors that accumulate glycogen appears promising as an indicator of tumor burden. As there is currently no biochemical marker for sarcomas, similar to the CEA and alpha-fetoprotein markers for some carcinomas, the excretion of $(G)_4$ offers a possible biochemical assay for monitoring tumor burden in selected patients.

Proposed Course: Measurements of $(G)_4$ excretion in patients with neuromuscular, metabolic, and neoplastic diseases will continue to further explore the utility of this assay and correlation with clinical status.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00511-03 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Carbohydrate Heterogeneity in Alpha Subunits of Human Polypeptide Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Nilsson	Visiting Scientist	LP	NCI
OTHER:	D.A. Zopf	Surgeon	LP	NCI
	D.W. Rosen	Senior Investigator	CE	NIAMDD
	B. Weintraub	Senior Investigator	CE	NIAMDD

COOPERATING UNITS (if any)

Clinical Endocrinology Branch, NIAMDD

LAB/BRANCH
Laboratory of Pathology

SECTION
Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Purified alpha subunits from human chorionic gonadotropin, TSH, FSH, and LH will be treated with neuraminidase and then subjected to alkaline borohydride degradation followed by trifluoroacetolysis. Oligosaccharides released by the alkaline borohydride step will be studied by gel filtration, methylation analysis and mass spectrometry of the permethylated oligosaccharide derivatives. Conditions for trifluoroacetolysis will be adjusted so as to destroy reducing amino sugars after release of oligosaccharides from chitobiosyl-asparagine linkages. Following removal of N-trifluoroacetyl groups from any remaining amino sugars in the mixture, oligosaccharides will be subjected to ion exchange chromatography to separate "high mannose" from "complex" type chains. The oligosaccharides obtained will be subjected to gel filtration chromatography, high voltage electrophoresis in borate buffer, and paper chromatography to investigate possible heterogeneity of carbohydrate chains. Fractions will be monitored by sugar analysis at each step.

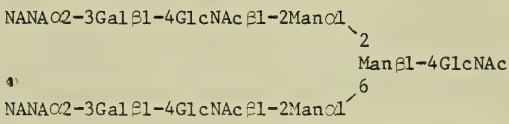
PHS-6040
(Rev. 2-81)

Project Description

Objectives: To investigate possible differences in structure of carbohydrate chains among alpha subunits of human polypeptide hormones.

Methods Employed: We are applying a recently developed method - trifluoroacetolysis - that hydrolyses the polypeptide portion of glycoproteins but leaves carbohydrate chains intact. Conditions can be adjusted so as to selectively destroy certain sugar residues such as reducing amino sugars. Heterogeneity of sugar chains will be investigated by gel filtration, high voltage paper electrophoresis in borate buffers, and analysis of monosaccharide components by gas chromatography and mass spectrometry after conversion to their alditol acetates. Further structural analysis will be carried out if sufficient material is available.

Major Findings: Human urinary hCG-alpha is heterogeneous in its carbohydrate portion. The molecule contains only asparagine-linked oligosaccharides, the largest of which has the following structure:



Oligosaccharide chains differ by the presence or absence of one or both non-reducing disaccharide termini (i.e., NANA 2-3 Gal- units). The relative proportions of the chains will be determined.

Significance to Biomedical Research and the Program of the Institute: If our examination of alpha subunits derived from different polypeptide hormones reveals differences in their patterns of glycosylation, these differences in carbohydrate structure may serve as a marker for the cell of origin for endocrine tumors that secrete alpha subunits. At present radioimmunoassays used to classify endocrine tumors on the basis of the hormones they produce identify beta subunits but cannot distinguish alpha subunits produced by different endocrine cell types.

Proposed Course: Model oligosaccharides are being prepared from the carbohydrate chain of transferrin by partial enzymatic hydrolysis. These oligosaccharides will be compared with the chains released from hCG to determine the type and extent of heterogenetiy in glycosylation of the hormone.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00523-02 LP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Complex Carbohydrate Released from Mammalian Cells by Trifluoroacetolysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Surgeon	LP	NCI
	B. Nilsson	Visiting Scientist	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Trifluoroacetolysis is a recently-developed method that releases oligosaccharides intact from glycoproteins and glycolipids. Carbohydrate chains released as a mixture from whole tissues, tissue fractions, or cells grown in culture, are easily recovered in nearly quantitative yield and reconstituted to their native form. Analysis of the majority of oligosaccharides containing six or fewer monosaccharide units is performed by combined gas chromatography and mass spectrometry of permethylated, N-trifluoroacetylated oligosaccharide derivatives. Analysis for certain specific oligosaccharides is carried out by radioimmunoassay using antibodies produced against purified oligosaccharides coupled to polypeptide carriers. It is anticipated that the repertoire of oligosaccharide chains produced by cells or tissues will reflect states of cellular differentiation and reveal potential cell surface markers.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To separate and identify the major oligosaccharide chains present in mammalian cells and to correlate the occurrence of specific oligosaccharide structures with states of cellular differentiation.

Methods Employed: Oligosaccharides are released by trifluoroacetolysis from whole mammalian tissues, tissue fractions, or cells grown in vitro. An aliquot of the mixture of sugar chains is reconstituted and fractionated by gel filtration chromatography. Fractions are analyzed directly by radioimmunoassay. The remainder of the mixture is treated so as to leave nitrogen atoms of amino sugars trifluoroacetylated and is reduced and permethylated. This preparation is analyzed by combined gas chromatography-mass spectrometry.

Major Findings: Due to delays in installation of a gas-chromatograph-mass spectrometer purchased in January 1980, this project has not progressed during 1980-81. Samples prepared during this year will be analyzed in the next few months.

Significance to Biomedical Research and the Program of the Institute: There is abundant evidence that cell surface carbohydrates can mediate specific binding of hormones, toxins, immunoglobulins, lectins, and other macromolecules. This project is designed to survey the similarities and differences in oligosaccharide patterns produced by cells at various stages of cellular differentiation in order to develop chemical and immunologic markers for cellular differentiation. In cases where major differences exist between normal and tumor tissues, oligosaccharides may be useful as tumor-specific markers.

Proposed Course: Investigations of oligosaccharides released from human tissues will continue using radioimmunoassay and GC/MS methodology developed for this purpose. A range of cell and tissue types will be tested including cells in culture that respond to "differentiating agents", fetal and adult tissues, and tumors.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00525-02 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of Oligosaccharides by Combined Gas Chromatography-Mass Spectrometry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Surgeon	LP	NCI
	B. Nilsson	Visiting Fellow	LP	NCI
	J. Cashel	Med. Tech.	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Separation of reduced and permethylated oligosaccharides by gas chromatography can be facilitated by the use of a fused silica capillary column 100 meters long coated with methyl silicon. The presence of N-acetylhexosamines in oligosaccharides increases retention time and decreases efficiency of separation. Transamidation of hexosamines by trifluoroacetolysis followed by reduction, removal of O-trifluoroacetyl groups and permethylation dramatically reduces the retention time of hexosamine-containing oligosaccharides and permits useful separation of oligosaccharides containing up to six monosaccharde units, regardless of how many of these are hexosamines. The mass spectra of permethylated oligosaccharides with N-trifluoroacetylated amino sugars show unexpectedly high abundances of mass ions containing the N-trifluoroacetyl group. As many of these ions are large, they provide useful information regarding oligosaccharide structure.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To develop methods for separation and analysis of oligosaccharides by gas chromatography and mass spectrometry.

Methods Employed: Following trifluoroacetolysis, oligosaccharides are treated with sodium borohydride and methanolic ammonia and finally are permethylated. N-trifluoroacetylated and permethylated oligosaccharide alditols are separated by gas chromatography using a fused silica capillary column 50 meters long coated with methyl silicon. The column effluent is passed without separation into a mass spectrometer.

Major Findings: Analysis of oligosaccharides as permethylated, N-trifluoroacetylated alditols can be accomplished by combined gas chromatography-mass spectrometry for molecules containing up to seven monosaccharide units including two hexosamines. Standards prepared by trifluoroacetolysis of purified glycolipids and glycoproteins with known carbohydrate structures enable identification of compounds according to retention time on GC under standard conditions and mass spectra. For example, relative retention times for some commonly-occurring oligosaccharides are as follows:

oligosaccharide	relative retention time
ganglio-N-tetraose	1.01
globo-N-tetraose	0.96
lacto-N-tetraose	1.05
lacto-N-neotetraose	1.07
lacto-N-fucopentaose I	1.38
lacto-N-fucopentaose II	1.28
lacto-N-fucopentaose III	1.27
lacto-N-difucohexaose I	1.47

Significance to Biomedical Research and the Program of the Institute: Structural analysis of oligosaccharides released from biological glycoconjugates usually requires purification and multiple analytical procedures to establish sugar sequence, linkage positions, and anomeric configuration. The gas chromatography/mass spectrometry method under development will permit a direct estimate of structural diversity in oligosaccharide mixtures and, in many cases, identification of oligosaccharides according to retention time and mass spectrum by comparison with standards. This method will be useful in many studies of carbohydrate structure, for example, it should provide a means of determining the repertoire of oligosaccharide structures present on cell surface membranes. The oligosaccharides of cell surfaces may be useful indications of cellular differentiation.

Proposed Course: Studies have been carried out on approximately thirty standard oligosaccharides derived from human milk, glycolipids, asparagine-linked chains of glycoproteins, and other sources. Additional oligosaccharides derived from human blood group substances, proteoglycans, and urinary oligosaccharides are under study.

Publications

1. Nilsson, B., and Zopf, D.A.: Gas chromatography and mass spectrometry of hexosamine-containing oligosaccharide alditols as their permethylated, N-trifluoroacetyl derivatives. Meth. Enzymol., vol. 83, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00529-02 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure of the Carbohydrate Portion of Human Haptoglobin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Nilsson	Visiting Scientist	LP	NCI
OTHER:	D.A. Zopf	Surgeon	LP	NCI
	G.C. Ashwell	Chief	LBM	NIAMDD
	M.E. Lowe	Staff Fellow	LBM	NIAMDD

COOPERATING UNITS (if any)

Laboratory of Biochemistry and Metabolism, NIAMDD

LAB/BRANCH
Laboratory of Pathology

SECTION
Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Haptoglobin is a plasma glycoprotein whose half time in the circulation of the rat is 19 days. When haptoglobin forms a complex with hemoglobin the complex is removed from circulation by the liver with a half time of only 4 minutes. Uptake of the complex by hepatocyte membranes is specifically blocked by glycopeptides derived from exhaustive pronase digests of haptoglobin and is inhibited by prior treatment of the complex with periodate or glycosidases. The haptoglobin complex does not inhibit uptake of asialo-orosomucoid by hepatocyte membranes, nor vice versa. Since the above evidence suggests that uptake of the haptoglobin-hemoglobin complex by hepatocyte membranes is mediated by a carbohydrate specific membrane receptor different from that responsible for uptake asialo-orosomucoid, we have undertaken structural analysis of the carbohydrate portion of haptoglobin.

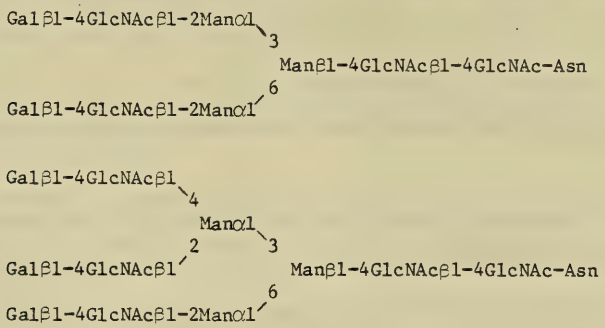
PHS-6040
(Rev. 2-81)

Project Description

Objectives: To determine the chemical structure of the carbohydrate portion of haptoglobin.

Methods Employed: Following sugar analysis of whole purified haptoglobin, the carbohydrate chains are released by trifluoroacetolysis. Further specific chemical degradative steps including Smith degradation, nitrous acid deamination, chromium trioxide oxidation, and selective acid hydrolysis produce products which are analyzed by sugar analysis, methylation analysis, and permethylation of oligosaccharides. Analyses at all stages are carried out by combined gas chromatography-mass spectrometry.

Major Findings: 1) Haptoglobin contains four asparagine-linked oligosaccharides per polypeptide chain, two with biantennary and two with triantennary structures. These include the following sugar sequences:



2) Fucose is present in the sequence Galβ1-4GlcNAc-
3
Fucα1

3) All galactose residues are substituted with sialic acid in the 3- or 6-positions. The ratio of 6-substituted to 3-substituted galactose is 4:1.

Significance to Biomedical Research and the Program of the Institute: Determination of the carbohydrate structure of haptoglobin may define the chemical structure responsible for specific uptake of the hemoglobin-haptoglobin complex by hepatocytes. As this uptake system appears to be functionally distinct from other glycoprotein uptake systems, it provides an opportunity for the study of independent function and regulation of two membrane bound receptors on the same cell.

Proposed Course: Structural analysis is in progress and will continue.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00549-01 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Hybridoma Antibodies to Oligosaccharide Haptens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.R. Schroer	Sr. Asst. Surgeon	LP	NCI
OTHER:	D.A. Zopf	Surgeon	LP	NCI
	A.K. Lundblad	Visiting Scientist	LP	NCI
	J.A. Fernandez	Biological Lab. Tech.	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hybridoma proteins (HP) with binding characteristics of high specificity and affinity for oligosaccharides would be invaluable reagents in the study of oligosaccharide localization, synthesis and excretion. Additionally, such antibodies might be used to purify or assess homogeneity of oligosaccharides from biological samples (of urine, plasma, tumors, etc.). High affinity characteristics of HP might be advantageously utilized for quantitative radioimmunoassays via isotope dilution techniques. Glycoconjugates of a urinary glucose-containing tetrasaccharide-Glcα1-6Glcα1-4Glcα1-4 Glc-linked to keyhole-limpet hemocyanin (KLH) have been used to examine the general feasibility and ease of this hybridoma approach to carbohydrate-structural analysis.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To examine the diversity and binding characteristics of hybridoma proteins (HP) constructed against the carbohydrate portions of glycoconjugates.

Methods Employed: A glucose-containing urinary tetrasaccharide, $(Glc)_4$, has been purified, phenethylamine derivitized and conjugated to keyhole-limpet hemocyanin for use as an immunogen in mice. Following hyperimmunization, spleen and lymph node cells were fused with PEG to a murine myeloma cell line and hybrids selected with HAT medium. Hybridomas were screened for desired antibody production, cloned *in vitro* and passaged *in vivo* to generate monoclonal antibodies.

Major Findings: 1. The antibody response of mice to $(Glc)_4$ is quite diverse, but of very small magnitude, 1-10 μg/ml. The hybridomas reflect this diversity but routinely generate 1-10 mg/ml of antibody (a 100-1000 fold increase). More than 20 such hybrids have been established.

2. The HP of these hybridomas are variously IgM, IgG, and IgA; show spectrotypic diversity by isoelectric focusing; additionally, they show considerable variation in affinity for the free oligosaccharide in a radioimmunoassay (RIA).

3. Further RIA comparisons are in progress to map the binding characteristics of each protein. Such an analytical approach is essential to confidently use these HP in chromatographic preparations of crude oligosaccharide mixtures.

Significance of Biomedical Research and the Program of the Institute: Structural analysis of important cell surface carbohydrates is greatly facilitated by use of antibodies as reagents that identify, purify and assay for specific structural determinants. Homogeneous antibodies prepared against synthetic immunogens bearing specific carbohydrate determinants provide a means to generate these reagents. Hybridoma antibodies against $(Glc)_4$ will be sought to replace currently-used rabbit serum antibodies for RIA analyses of urinary excretion of this oligosaccharide.

Proposed Course: The utility of this model system to enable one to assay for specific oligosaccharides will be further evaluated as a prelude to analysis of more complicated oligosaccharide structures.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00521-11 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Epithelial-Mesenchymal Interactions in Neoplastic Development

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.J. Dawe	Chief, Comparative Oncology Section - Med. Officer (Path.)	LP	NCI
OTHER:	S.E. Fisher	Post-doctoral Fellow	LP	NCI
	W.D. Morgan	Biologist	LP	NCI
	J.E. Williams	Biologist	LP	NCI
	M.A. Israel	Senior Investigator	I	LBV
	M.L. Meltzer	Microbiologist	I	IRP
	S.K. Arya	IPA (Roswell Park Mem. Inst.)	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Comparative Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 1/2	2 1/2	3

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project currently investigates 3 aspects of polyoma tumors arising from epithelial cells in mice: 1) Interactions between Py-transformed salivary epithelium (PTSE) and normal fetal mouse dermis. It has been found that PTSE induces melanin synthesis in normal dermal melanoblasts. Pigmented melanocytes then transfer melanin granules to PTSE. Normal salivary epithelium interacting with normal dermis was, predictably, found able to form complete hair follicles, sebaceous glands, and hair. 2) Isozyme studies of Py-induced tumors of several epithelial types showed profiles for 30 enzyme systems to be constant for each organ of origin, but different from organ to organ except for salivary and mammary gland, which had identical profiles. Spontaneous mammary and salivary gland tumors had isozyme profiles differing within the respective sets and differing from polyoma tumors of corresponding organ origin. 3) Experiments in progress investigate the relationship between Py genetic variants and organ tropism. Combined evidence suggests Py transformation in epithelia is mediated through a normal cell proliferation mechanism utilized within a specific group of organs, normally operative via mesenchymal-epithelial interaction.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Emphasis has shifted during the past year. Previous efforts had been mainly toward determining the effects of alterations of micro-environment (epithelial-mesenchymal interactions) on the end result of polyoma virus-induced transformation of salivary epithelium. Those studies have largely been completed and the results have been useful in developing a concept that will be subjected to test in collaboration with Dr. Mark Israel, working in viral genetics.

In oversimplified form, the concept is that one or more of the T antigens coded for by Py controls the organ specificity of the transforming action of the virus, in mice. It is already known that middle T antigen is essential for initiation and probably for maintenance of transformation. Based on our earlier work, it is now postulated that one or more of the Py T antigens either itself simulates a mitogen normally produced by the mesenchymes of the various organs involved, or the T antigens mediate continuous production of such a mitogen. In either event, the transformed epithelium would acquire independence of its former control by mesenchyme mitogen factor.

Initially, the experiments in progress ask a broader question: Given 2 or more strains of Py, each with different organ-range specificity, what differences can be found in their genetic structure at the molecular level and through what mechanism (e.g. T antigens, other more proximal mitogens) are these differences expressed? A corollary question is: Does Py produce or cause to be produced in the transformed epithelial cell a mitogen or growth factor that is similar to or identical with a factor produced by normal mesenchyme? Thus the experiments are designed to generate information that is fundamental not only to viral oncology, but also to developmental biology.

Methods Employed: Already available are 2 strains of polyoma virus for which we have preliminary evidence of differences in organ specificity relevant to transforming ability. These are LID_1 and 2PTA. Both are derived from the same original virus isolation, but the former has been passed through newborn mouse kidney *in vivo* for many generations, while the latter has been passed through mouse lymphoma cells ($P388D_1$) *in vitro* for many generations.

Dr. Israel has plaque-cloned both virus strains on mouse embryo cells. Two clones of each strain have each been divided into 2 stock sources. The resulting 4 stock sources have been grown on each of 2 cell types: mouse kidney and mouse $P388D_1$ cells. Each of the resulting 8 stocks has been frozen in liquid N_2 and has been plaque-titered. Equal doses of each stock are being inoculated into newborn C_3H/BiDa and NIH mice. The mice are observed for tumor development and other viral effects such as runting and lethality. At present it is already evident that strain LID_1 has much greater lethality in C_3H/BiDa mice than strain 2PTA. Strain 2PTA has induced mammary, hair follicle, thymic, adrenal medullary, and salivary tumors in C_3H/BiDa mice. Observation of NIH mice has been too brief to make projections.

When observations on about 800 mice over a period of one year have been completed, they are expected to provide a reliable picture of the differences

in biological action of the 8 stocks of virus. Meanwhile Dr. Israel is characterizing the virus stocks as to genetic homogeneity on the basis of electropherograms of endonuclease digests of viral DNA, and will in the future examine them for differences in T antigens and, ultimately, in base-pair sequences in the viral genomes, provided that at least some of the stocks are sufficiently homogeneous to allow meaningful interpretation. From these sets of information, correlation will be made between biologic activity and the genetic structure of the respective virus strains.

When it becomes possible to obtain antibodies specific for the various polyoma T antigens and for the 53K and other cell-associated proteins of transformed cells, immunoperoxidase procedures will be set up to determine whether any of these antibodies is cross-reactive with proteins in fetal salivary mesenchyme.

Major Results: During the past year attempts to transfect salivary epithelium with fragments of Py DNA (early- and late-translating fragments as well as whole Py DNA) prepared by Dr. Israel have been unsuccessful as judged by failure to obtain any tumors from salivary epithelial cells exposed to any of the preparations. Efficiency of transfection is apparently too low to give positive results in our system, which contains only $0.5 - 1.0 \times 10^4$ salivary cells in a non-monolayer situation. We will probably not renew efforts along this line unless a more efficient transfection method is found.

Work reported in abstract last year on the ability of Py-transformed salivary epithelium to interact with dermis/melanocytes in a manner comparable to hair follicle epithelium has been nearly concluded. The chief observation was that the Py-transformed salivary epithelium induced maturation and melanogenesis is normal dermal melanoblasts. Transfer of melanin granules from dermal melanocytes to transformed salivary epithelium then followed. From this it was inferred that normal salivary epithelium should have hair-forming capability. This proved to be so. When mesenchyme-free salivary epithelium was combined with epidermis-free dermal mesenchyme and grown in organ culture, hair follicles complete with hair shafts and sebaceous glands developed. This observation seems to offer experimental support for the postulation that accessory salivary glands of the oral cavity have some of the potentialities of hair follicles, as seen clinically when Fordyce's spots (sebaceous metaplasia) occur in middle-aged and elderly people. It is planned to publish a more complete report on this work within the year.

The study on mammary tumors induced in athymic (nude) mice inoculated with Py at 6 weeks or more of age is also near completion and has been extended by Dr. Arya's DNA and RNA probe analysis of the tumors. Dr. Arya found no increase of MTV provirus genome in the tumor cell DNA, but an average of 10-100 Py genomes per tumor cell. His findings suggest that some complete virus replication occurs in these tumors and that although some virus appears to be integrated, much is not. A paper characterizing the morphological and biological properties of these tumors, including their de novo genesis from terminal mammary ducts in the absence of hyperplastic alveolar nodules, is in preparation. Dr. Arya's work will be reported either separately or in a joint publication.

An unanticipated observation was made this year by Mr. Morgan in the course of now routine Py transformations of salivary epithelium grown on millipore membranes in perfusion chambers. He noted continuous growth of salivary epithelium in an uninfected control culture. This had not been seen before in similar control cultures. Tissue culture infectivity tests for Py virus in this anomalous culture have been repeatedly negative. At this time it is not known whether the continuous epithelial growth signifies "spontaneous" transformation or whether the epithelium retains the properties of normal salivary epithelium. Morphologically it appears normal. Until now Mr. Morgan has not succeeded in getting the epithelium to grow on glass surfaces, but he has been able to subculture onto millipore membrane. This is a slow and tedious way to obtain sufficient numbers of cells for experimental uses, but we hope eventually to be able to have sufficient cells to test for the developmental potentialities of this apparently normal epithelium. In particular it will be of interest to see whether it can undergo normal morphogenesis when combined with normal salivary mesenchyme and transplanted to a newborn mouse. If the line can be maintained it will also be very useful as a constant source of epithelium for transformation by Py virus, and possibly as a cell line on which to propagate Py virus. Py virus grown exclusively on salivary epithelium would be extremely interesting to study from the standpoint of biological properties in relation to genetic constitution.

Mr. Morgan has also continued his investigation of myoepithelial contraction in organs containing myoepithelium. He has turned to study of mammary gland and has developed a technique for placing post-partum lactating mouse mammary glands in perfusion chamber organ culture, where they can be photographed with time-lapse cinematographic methods. His initial results show contractions comparable to those reported earlier in salivary gland. These are present during the first few days after isolation, and then appear to be lost either because of nutritional problems or because of hormonal and other physiological factors present in lactating mice but not in organ cultures.

Dr. Fisher has continued her study of the isozymes of polyoma virus-induced tumors of the mouse. The additional salivary gland and mammary gland tumors she has analyzed show the same patterns of isozyme changes as the tumors previously studied. Mammary tumors produced by polyoma virus in nude mice with a C_3H/Hen background have isozyme profiles identical to those induced by polyoma virus in nude mice with an NIH background. She has continued to expand the number of enzymes she uses in analyzing the isozyme profiles of the tumors. By a stain to detect orthophosphate, she has added a number of enzymes involved in nucleotide metabolism and amino acid synthesis.

Dr. Fisher has continued to analyze the TMI tumors that result from the *in vitro* transformation by polyoma virus of isolated salivary epithelium, isolated salivary mesenchyme and salivary epithelium separated by a millipore filter from salivary mesenchyme. The TMI tumors continue to show considerable isozyme variability when compared to the *in vivo* induced tumors. This variability cannot simply be explained by the multiplication or loss of *large* chromosome segments, though the TMI tumors are aneuploid, approaching tetraploidy. For example, the IDH 1 and peptidase 3 loci are 24 units apart on chromosome 1. TMI tumors show increased IDH 1 activity but no change

in peptidase 3. Four of the markers studied are on chromosome 7 in the following order: 6pi---13 units---Ldha---16 units---Mod2---Pep4 (The exact map distance between Mod 2 and Pep 4 is not known). The TMI tumors generally show no change in GDI, an increase in LDH subunits, an absence of mitochondrial malic enzyme (Mod 2) and no change in peptidase 4. The changes in isozymes that are observed may be due to duplications or deletions in small segments of chromosomes, changes in location of genetic material resulting in changes of gene expression, or alterations in gene expression unrelated to positions of genes on chromosomes.

Dr. Fisher has studied the isozyme profiles of two spontaneous tumors for comparison with the polyoma virus-induced tumors. One of these is a series of mammary tumors that occur by one year in heterozygous (C_3H Hen/Nu) females that have been repeatedly bred. These Mam-Hes tumors are histologically somewhat variable showing mixtures of type A and B tumors according to Dunn's classification and having solid, glandular, and acinar characteristics. With serial transfer in C_3H/Hen mice a more uniform tumor is maintained. In contrast to the uniformity of isozyme pattern for the polyoma-induced mammary tumors, the Mam-Hes tumors are variable for a number of enzymes including: mannose phosphate isomerase, glycerol-3-phosphate dehydrogenase, hexokinase, isocitrate dehydrogenase, creatine kinase, xanthine dehydrogenase, and catalase.

The second spontaneous tumor that Dr. Fisher has studied is a myoepithelioma of the salivary gland in Balb/c mice. Dr. Daniel Malamud has provided three independently-arising spontaneous myoepitheliomas. These tumors show both similarities and differences of isozyme patterns when compared to polyoma-induced salivary gland tumors. Like the polyoma salivary tumors the myoepitheliomas exhibit the PGM 2 isozyme not found in normal submandibular gland. However, the myoepitheliomas do not express the placental alkaline phosphatase found in polyoma salivary tumors. The myoepitheliomas differ among themselves in isozyme repertory, most notably for lactate dehydrogenase.

The comparisons to spontaneous tumors highlight the uniformity of isozyme profiles for a given histotype of polyoma virus-induced tumor. This consistency suggests a specific interaction of the polyoma virus DNA with the genome of the cell being transformed.

A third type of comparison has been made between the isozymes of the polyoma virus induced tumors of the submandibular gland and the isozymes of the developing submandibular gland. Submandibular glands collected at 13, 14, 15, 16, 17, 18 days gestation 1, 7, 30, and 60 days after birth were analyzed. The PGM 2 isozyme detected in all salivary gland and TMI tumors but not in male or female adult salivary glands is found from 13 days gestation to 7 days after birth. The expression of PGM (phosphoglucomutase) 2 in tumors is evidently a return to a fetal pattern of gene expression. Not all of the isozymes studied show a switch to a fetal pattern in tumors. While salivary tumors show a preponderance of LDH A subunits, all fetal stages show nearly equal expression of Ldh A and B genes. A number of enzymes (malate dehydrogenase, glucosephosphate isomerase, aconitase) that show no differences in adult glands and tumors are expressed

early and continuously throughout development. Stage specific isoyzmes were seen for mannose phosphate isomerase and phosphoglucomutase; these have not been detected in tumors. For isozymes localized to the cytosol or mitochondria, examples of concordant and discordant expression were seen. The comparison to fetal stages indicates that the pattern of gene expression in tumors is probably better described as unbalanced rather than strictly fetal or primitive. Dr. Fisher plans to examine the isozyme repertory of fetal stages of other tissues susceptible to polyoma virus including thymus and kidney.

Significance to Biomedical Research and the Program of the Institute: These studies, directed mainly toward the cellular responses of epithelial tissues to Py virus during cell-virus transformational processes, have led to a point of convergence with the findings and thinking of virologists and viral geneticists. On the one hand, our studies have led us to formulate an hypothesis that Py transformation occurs as a result of viral activation (or mimicry) of a mechanism present in the normal epithelial organs affected. The factor activated or mimicked is normally produced and controlled by mesenchymal cells that induce growth of epithelium. It has not been isolated, purified, and chemically characterized, and its existence is only presumed at present on the basis of 1) the growth-stimulating effect of normal mesenchyme interacting with epithelium; and 2) the growth-stimulating effect of Py salivary tumors acting both in vivo and in vitro on normal salivary epithelium. The isolation and characterization of this factor is obviously crucial to the proof of the hypothesis.

On the other hand, virologists using the methodology of molecular biology have identified viral gene products (T antigens) and host-cell gene products (53K and several other proteins under investigation by Ito and others) that deserve consideration as either the mimicker of or the actual mesenchyme inductive (MI) factor.

It would seem appropriate at this time to coordinate our efforts with those of viral oncologists and molecular pathologists to determine whether the MI factor is identical with or similar to any of the viral or cellular gene products mentioned above. Work could be greatly accelerated by addition of a skilled protein chemist to our group, so that the MI factor could be isolated and characterized. At that point immunological methods could be used to determine whether the MI factor is similar to or identical with T antigens or transformed host cell proteins. Another approach, already open, would be to prepare antibodies vs. each of the known T antigens and host-cell antigens and use these in PAP tests to detect identical or cross-reacting antigens in normal fetal salivary mesenchyme. To do this, we would need the collaboration of those who can prepare and provide the antigens in question.

The implications to the viral oncology program of NCI are rather obvious. There seems little doubt that the molecular mechanisms of Py oncogenesis will ultimately be worked out in detail by "VOMPs" at NCI and elsewhere. However, it also seems their efforts could be accelerated by making use of the biological materials we have developed. Certainly there is little or no thought being given elsewhere to the possibility that an understanding of events in Py tumorigenesis in the salivary gland may answer some of the most engaging questions in developmental biology.

Publications

1. Fisher, S.E., and Dawe, C.J.: Isozyme analysis of polyoma virus-induced tumors in mice. Cancer Bull. 32: 61-65, 1980.

2. Dawe, C.J.: Polyoma tumors in mice and X cell tumors in fish, viewed through telescope and microscope. Nakahara Memorial Lecture. In Takayama, S. and Sugimura, T. (Eds.): Phyletic Approaches to Cancer. Proceedings of the 11th International Symposium of The Princess Takamatsu Research Fund, Tokyo, 1980. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00538-16 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Identity of X Cells in "Tumors" of Fishes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.J. Dawe	Chief, Comparative Oncology Section - Med. Officer (Path.)	LP	NCI
OTHER:	C.M. Poore	Biologist	LP	NCI
	A.J. Garvin	Surgeon	LP	NCI
	S.E. Fisher	Guest Worker	LP	NCI
	M.C. Habbersett	Biologist (Cytol.)	LP	NCI

COOPERATING UNITS (if any)

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SECTION
Comparative Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

X cell lesions (papillomas and pseudobranch tumors) have been reported in the literature to involve fishes of 3 Orders (Pleuronectiformes, Gadiformes, and Perciformes) covering about 40 species. For some 70 years these lesions have been considered neoplastic or pre-neoplastic. In imprint preparations in this study, X cells stained by Wright's, Feulgen, and a DNA-binding fluorescent dye (bisbenzimidazole), the features of the mitotic cycle of X cells were visualized in detail and found to be closely similar to those of hartmannellid amoebae. Mitoses of X cells differed markedly from mitoses of host fish cells. In multinucleated plasmodia-like forms, mitoses of the several nuclei in a given cell were synchronized. Isozyme studies revealed starch gel electrophoresis bands extractable from X cells but not from host fish cells, active in at least 4 to 6 enzyme systems. Flow fluorometric quantitation of DNA showed the X cell population to be sharply distinct from that of host fish cells, with X cell/fish cell DNA ratio of about 1:3. It was concluded that X cells are parasitic rather than neoplastic in nature.

Project Description

Objectives: The title of this project has been changed from "Phylogenetic Aspects of Neoplasia" because activities in this area have been reduced as indicated by the new title. The PI continues to serve as Project Officer for the contract with the Smithsonian Institution to operate the Registry of Tumors in Lower Animals, but his scientific contribution is limited to diagnostic assistance. In October 1981, the contract funding will be the responsibility of DCCP and will be administered through a contract managed by EPA on funds transferred from DCCP. The PI will continue as Project Officer, with Dr. John A. Couch of EPA as Co-Project Officer.

The objective of the study of X cell lesions in fishes is to determine whether X cells are neoplastically or otherwise transformed cells of host origin, or are xenogenous cells of parasitic nature.

The methods used were described in last year's report and consist of 1) standard histological examination of lesions; 2) electron microscopy; 3) cytochemical procedures performed on sections and imprint preparations; 4) flow microflourometry for determination of relative quantities of DNA per cell; 5) isozyme analysis using 26 enzyme systems; 6) immunoperoxidase techniques.

Major Findings: Histological and ultrastructural characteristics of the pseudobranch "tumors" in Pacific cod were published in an earlier report (J. Natl. Cancer Inst. 59: 377-398, 1977), and the more recent observations are included in a report in press listed below. In summary, in imprint preparations of X cells stained by Wright's, Feulgen, and a DNA-binding fluorescent dye (bisbenzimidazole), the features of the mitotic cycle of X cells were visualized in detail and found to be closely similar to those of amoebae in the family Hartmannellidae. Mitoses of X cells differed markedly from mitoses of any known vertebrate. In multinucleated plasmodia-like forms, mitoses of the several nuclei in a given cell were synchronized, but did not take the form of multipolar figures, as in neoplasms.

Isozyme studies by Dr. Fisher revealed starch gel electrophoresis bands extractable from X cells but not from host fish cells, active in at least 4 to 6 enzyme systems. Flow fluorometric quantitation of DNA per cell showed the X cell population to be sharply distinct from that of host fish cells, with X cell/fish cell DNA ratio of about 1:3. Comparisons of X cells and fish cells by immunoperoxidase technique have not been completed. These are being done in collaboration with Dr. A.J. Garvin, now at the University of South Carolina Medical School.

Significance to Biomedical Research and the Program of the Institute: X cell lesions have been reported in the literature to involve fishes of 3 Orders (Perciformes, Gadiformes, and Pleuronectiformes), covering approximately 40 species. For some 70 years these lesions have been considered to be neoplasms and much effort has been invested by cancer researchers. Although the life cycle and exact taxonomic position of X cells remain to be worked out, our findings leave no doubts that they are xenogenous organisms. They may remain of interest to cancer researchers to a limited degree, since they do appear to

elicit a mild proliferative response (non-neoplastic) in the epithelia in which they reside.

This project will be concluded after completion of the immunologic aspect of the work.

Publications

1. Dawe, C.J.: Polyoma tumors in mice and X cell tumors in fish, viewed through telescope and microscope. Nakahara Memorial Lecture. In Takayama, S. and Sugimura, T. (Eds.): Phyletic Approaches to Cancer. Proceedings of the 11th International Symposium of the Princess Takamatsu Research Fund, Tokyo, 1980. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00533-08 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Study of Virus Cell Interaction and Biological Characterization of Human Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.C. Costa	Chief, Surgical Pathology & Postmortem Section	LP	NCI
OTHER:	A.S. Rabson	Acting Chief, Laboratory of Pathology	LP	NCI
	C.L. Yee	Biologist	LP	NCI
	T.S. Tralka	Biologist	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4 1/2	3	1 1/2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Viruses belonging to the human herpesvirus family induce a receptor for the Fc portion of IgG during their lytic cycle. We are studying the receptors induced by herpes simplex virus in mammalian cells. We are also studying the induction of Fc receptors in lymphoblastoid cell lines by Epstein-Barr virus. Definition and characterization of the receptor will allow us to gain insights on the possible biological role of the receptor during lytic infection.

We are also studying biological properties of human tumors carried in nude mice and of tissue culture cell lines derived thereof. The synthesis of specific tumor products that serve as markers and their regulation is being studied.

PHS-6040
(Rev. 2-81)

Project Description

Virus-cell Interaction

Studies on the induction of Fc receptors by Epstein-Barr virus (EBV) have shown that whereas the P3HR-1 bearing cells exhibit an increase in Fc-R activity when induced, B-95-8 cells fail to do so. Comparison of the P3HR-1 strain of virus with the B95-8 and AG-1 strains is in progress and focuses on the ability of the strains to generate Fc receptors and on the presence or absence of an Fc-R at the virion envelope.

Ultrastructural studies of human xenografts in nu/nu mice have shown the presence of intercisternal A particles (IAP) in the human cells. This is the first time that IAP presumably of mouse origin have been seen in human cells with the exception of an isolated report by Sarah Stewart.

Publications.

See Report Z01 CB 00853-28 LP for publications.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00535-06 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biologic and Virologic Characterization of Breast and Prostate Cancer Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S.K. Arya IPA (Roswell Park Memorial Institute) LP NCI

COOPERATING UNITS (if any)

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Laboratory of Pathology

SECTION
Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cultures of a single cell clone of mouse mammary tumor cells ($Mm5mt/c_1$) that initially synthesized only MMTV (type B retrovirus) produced increasing amounts of type C retrovirus during passage in culture with some reduction in MMTV production. This was accompanied by a marked increase in the synthesis of type C viral RNA but not by a decrease in MMTV RNA. Mouse interferon inhibited the production of both viruses equally without a significant effect on intracellular concentrations of two viral RNAs. The restriction analysis of polyoma virus genome in nude mouse mammary tumors showed that viral genomes were organized as tandem repeats integrated in the cell genome. Some of the tumors also contained free copies of viral DNA and some viral DNA molecules had deletion covering 0.12-0.18 map units. A comparison of the sequence complexity and diversity of mRNA in cultured human breast and prostate carcinoma cells showed that about 80% of the sequences were held common. The abundant mRNA species of one cell were less frequently represented in the other cell but only by a factor of two to four.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To investigate the regulation of viral and cellular gene expression and their modulation by hormones and other chemical agents.

Methods Employed: The major methods employed include molecular hybridization, cell free protein synthesis, and southern and northern blotting and S-1 mapping.

Major Findings: Murine System. In a previous study we showed that continuous culture of mouse mammary tumor cells ($Mm5mt/c_1$) resulted in the production of increasing amounts of type C retrovirus (ecotropic) accompanied by a decline in type B retrovirus (MMTV) production. It was possible that this was due to the selection of different subpopulations of cells during passage. Single cell clones derived from $Mm5mt/c_1$ cells that initially synthesized only type B retrovirus also produced increasing amounts of type C retrovirus during passage in culture. This was accompanied by a marked increase in the intracellular concentration of type C retroviral RNA. Thus, type C retrovirus production was probably due to the activation of endogenous type C viral genes. A decline in the production of type B virus during passage was also noted but this has not yet been quantified.

Mouse interferon strongly inhibited the production of both type B and type C retroviruses by cloned $Mm5mt/c_1$ cells. It did not affect significantly the intracellular steady-state concentration of either type B or type C retrovirus RNA. This may be consistent with the notion that interferon inhibits virus production by blocking their budding through the cell membrane. This, however, posses an apparent paradox. The accumulation of virus particles on the cell membrane would be expected to result in the increased intracellular concentration of RNA (and other subviral components) in interferon-treated relative to control cells. It is possible that the synthesis and degradation of viral RNA is kinetically regulated in interferon-treated cultures. Alternatively, the complexity of viral RNA synthesized in interferon-treated cells may not be identical with that in untreated cells.

Polyoma virus inoculation of nude mouse lead to the development of mammary tumors rather than the more common parotid gland tumors as is the case for normal mouse. This was apparently not due to the activation of the endogenous mouse mammary tumor virus. We did not detect any amplification of MMTV genomic sequences or their transcription in mammary tumors compared with normal tissues. The mammary tumors contained multiple copies of polyoma virus genome per cell. The restriction analysis of genomic DNA from several tumors gave the following results: (i) Tumors contained tandem repeats of integrated viral genomes; (ii) Some tumors also contained free viral DNA molecules; (iii) Two deleted viral genomes (integrated and unintegrated) were detected in some tumors. In some DNA molecules the deletion covered 0.12-0.18 map units and in other molecules the deletion was larger. (iv) One tumor may contain only deleted genome integrated in the cell genome. However, it has not been ruled out that this tumor may contain one or a very few complete viral genomes also.

Human System. The sequence complexity and abundance frequency of mRNA in cultured human breast and prostate carcinoma cells was compared. Of the 50-60,000 kb sequence complexity, about 80% of the sequences were shared between the two cell types. Nearly all of the abundant sequences of one cell type appeared to be present in the second cell type. The abundant sequences of one cell (e.g., breast) were present in reduced abundance in the second cell (e.g., prostate) but only by a factor of two to four. This is to be compared with reported sequence distribution of normal tissues in vivo. In the case of mouse, abundant mRNA species of one tissue (e.g., liver) are reportedly present in orders of magnitude lower abundance in another tissue (e.g. kidney). Thus, this abundance sequence specificity was not observed for human breast and prostate cells. It is not clear if this is due to culturing or neoplastic origin of these cells or both.

Significance to Biomedical Research and the Program of the Institute: A delineation of control elements regulating viral and cellular gene expression may provide insights for understanding malignant transformation. An investigation of modulation of viral and cellular functions by hormones and other chemical agents may lead to the development of new diagnostic and therapeutic modalities.

Proposed Course: Murine System. Interferon strongly inhibits the production of type B and type C retroviruses by mouse mammary tumor cells. It has no significant effect on the intracellular concentrations of viral RNAs. One possibility is that the complexity of viral RNA synthesized in interferon-treated cultures is not identical with that in control cultures. This will be tested by the techniques of modified northern blotting and S-1 mapping. Initially poly(A)-selected RNA from treated and control cultures will be electrophorized in agarose gels, blotted to nitrocellulose filters and hybridized with viral probes. Additionally, poly(A)-selected RNA will be hybridized with cloned viral DNA, digested with S-1 nuclease and analyzed by agarose gel electrophoresis. If necessary, viral specific RNA will be pre-selected by hybrid selection procedures.

The organization and expression of polyoma virus genome in nude mouse mammary tumors will be further analyzed. Attempts will be made to obtain cultured cells and clones from these tumors particularly from a tumor which contains predominently, if not solely, deleted polyoma virus genome lacking sequences coding for carboxy end of large T-antigen. The expression of T-antigens in these cells and their transformed phenotype will be analyzed to delineate the role of T-antigens in transformation. Of additional interest will be to determine if the free viral DNA molecules observed in some tumors will be retained or replicated in cultured cells along with their transformed phenotype.

Human System. (a) Breast cancer cells. It remains possible that the expression of differentiated genes is regulated at the level of processing or translation of mRNA. Studies will be initiated to determine if these cells synthesize alpha lactalbumin and casein RNA with or without concomitant production of the corresponding proteins. We are particularly interested in analyzing prolactin-caused modulation of these genes and

their products. (b) Prostate cancer cells. We have obtained human prostatic cancer cells that produce significant amounts of specifically prostatic acid phosphatase. In addition, these cells when injected into nude mice produce tumors that secrete large amounts of prostatic acid phosphatase. Studies will be initiated to isolate mRNA for prostatic acid phosphatase. Starting with nascently labeled polysomes precipitated with prostatic acid phosphatase antibody, RNA will be enriched for desired mRNA by sizing, in vitro translation and immunoprecipitation.

Publications

1. Arya, S.K., and Young, N.A.: Synthesis in high yield of complimentary DNA of retroviral RNA. Prep. Biochem. 10: 483-493, 1980.

2. Arya, S.K., Czarniecki, C.W., and Friedman, R.M.: Interferon induced inhibition of mouse mammary tumor virus production. J. Interferon Res. 1: 147-154, 1980.

3. Strauchen, J.A., Arya, S.K., Tralka, T.S., Engel, L.W., and Young, N.A.: Search for retrovirus-like particles in human breast cells in culture. Cancer Res. 40: 3880-3885, 1980.

4. Arya, S.K.: Changes in retrovirus expression in mouse mammary tumor cells. Cancer Res. 41: 1579-1584, 1981.

5. Arya, S.K., and Gordon, B.: Effect of interferon on the synthesis of mouse mammary tumor virus (type B) and murine leukemia virus (type C) in the same culture. J. Gen. Virol. 53: 383-387, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00542-04 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on Oncogenic Primate DNA Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Howley	Senior Surgeon	LP	NCI
OTHER:	M.-F. Law	Visiting Fellow	LP	NCI
	F. Rentier-Delrue	Visiting Associate	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study involves the characterization of the primate polyomaviruses BK, JC, SV40, STMV, and SA12. Each of these polyomaviruses is oncogenic in hamsters and has been demonstrated to be capable of transforming certain rodent cells in tissue culture. BK and JC viruses are human polyomaviruses first described as isolates from human beings in 1971. While BK virus has not been clearly associated with any human disease, JC virus has been repeatedly isolated from the diseased brains of patients with progressive multifocal leukoencephalopathy (PML). The purpose of this work is to better characterize the human polyomaviruses BK and JC both in terms of their molecular characteristics as well as their biological properties. An understanding of how they interact with human cells will provide a better understanding of what pathogenic roles they may play in humans and what role, if any, they may have in human malignancy.

Project Description

Objectives: Our recent efforts have focused mainly on the human polyoma-virus JC. This virus is difficult to propagate in tissue culture and therefore to provide unlimited and standardized molecular reagents, it was necessary to clone the genomes of the prototype Mad-1 strain of JC virus in E. coli using the plasmid vector pBR322. We've been interested in examining the DNA of JC virus directly in human brains and comparing it to DNA from virus which has been propagated in human glial cells. We have attempted to analyze the genomic structure of a variety of isolates of JC virus to determine how much heterogeneity normally occurs in the natural infection of humans with this virus and to see how much variation there is among different isolates of this virus. Our current studies are designed to understand the genetic organization of the genome and to evaluate the role of this virus in human neoplasia.

Methods Employed: Molecular cloning, nucleic acid hybridization, restriction endonuclease analysis, tissue culture, and DNA transfection.

Major Findings: 1. The restriction endonuclease pattern of the molecularly cloned JC genome (Mad-1 strain) is similar to that of the DNA propagated in tissue culture in primary human fetal glial cells. A manuscript describing the cloning of the Mad-1 strain of JC DNA has been published in the Journal of Virology (see below). 2. We have purified JC DNA directly from two human PML brains utilizing the differential salt precipitation technique described by Hirt. The DNA present in these two brains is quite homogeneous in size, although in one brain there is a single subpopulation of viral DNA molecules which is slightly smaller in size and has lost the Eco RI site. The Eco RI site is located in the late region of the JC viral genome and presumably, therefore, this defective molecule contains a deletion in the late region. 3. These two new viral DNAs have been cloned in pBR322 independently at their single Bam HI sites and single Eco RI sites. These DNAs have been mapped using restriction endonucleases and are very similar in their structure to that of the Mad-1 strain indicating that genomes of different isolates of the JC virus are quite similar. 4. Despite these similarities, slight differences were noted in the restriction patterns of some of the multi-cut enzymes and a region of variance among the JC genomes was found to map in the non-coding region to the late side of origin of replication. A manuscript describing the analysis of JC DNA in human PML brains, its molecular cloning, and a comparative analysis of the restriction maps is in press in the Journal of Virology.

Significance to Biomedical Research and the Program of the Institute: JC virus is a known human pathogen and is capable of transforming a variety of rodent cells. A better understanding of the molecular biology of this virus will allow an evaluation of whether this virus has a role in human neoplasia.

Proposed Course: 1. To analyze the genetic organization of the genome. 2. Analyze by immunoprecipitation the viral proteins associated with transformation in early gene expression. 3. Direct sequencing of the DNA origin of replication, "agnogene" region, early and late transcriptional promoters, and early gene segments associated with transformation. 4. Use peroxidase anti-peroxidase techniques for evaluating sections of PML brains

for the presence of known viral antigens.

Publications

1. Howley, P.M., Rentier-Delrue, F., Heilman, C.A., Law, M.-F., Chowdhury, K., Israel, M.A., and Takemoto, K.K.: Cloned human polyomavirus JC DNA can transform human amnion cells. J. Virol. 36: 878-882, 1980.

2. Rentier-Delrue, F., Lubiniecki, A., and Howley, P.M.: Analysis of JC virus DNA purified directly from human PML brains. J. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00543-03 LP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Characterization of the Papillomaviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Howley	Senior Surgeon	LP	NCI
OTHER:	M.-F. Law	Expert	LP	NCI
	C.A. Heilman	PHS Fellow	LP	NCI
	N. Sarver	ACS Fellow	LP	NCI
	D. Lowy	Medical Director	D	NCI
	S. Schlegel	Expert Consultant	LEP	NCI

COOPERATING UNITS (if any)

Dermatology Branch, NCI; Laboratory of Experimental Pathology, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

There are to date eight recognized human papillomaviruses (HPV) and five bovine papillomaviruses (BPV). Each of the human papillomaviruses and bovine papillomaviruses are associated with distinct clinical entities including common warts, condyloma accuminata, and laryngeal papillomatosis in humans, and esophageal papillomatosis and cutaneous fibropapillomas in cattle. To date none of the papillomaviruses has been successfully propagated in tissue culture and consequently little is known of their molecular biology. A subset of papillomaviruses, however, are associated with carcinomas in their natural hosts. These include two of the human papillomaviruses: type 6, which has an epidemiological association with carcinomas arising in condyloma accuminata; and the type 5 human papillomavirus, which is associated with squamous cell carcinomas in patients which epidermodysplasia verruciformis. In cattle the esophageal papillomatosis is associated with alimentary tract carcinomas in those cattle which feed on bracken fern. Bovine fibropapillomas are caused by a virus (BPV-1 or BPV-2) which are capable of inducing tumors in hamsters as well as transforming certain mouse cells in tissue culture.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: 1. To clone the various papillomavirus genomes in bacterial systems in order to generate standardized nucleic acid reagents. 2. To analyze the molecular biology of the bovine papillomaviruses and the human papillomaviruses using whatever in vivo and in vitro systems are available. 3. To localize and characterize the transforming regions of various known bovine papillomaviruses and to determine if the corresponding regions are expressed in human papillomaviruses which have persistently infected rodent or human cells. 4. To develop a tissue culture system for the propagation of the papillomaviruses. 5. To analyze what, if any, role the human papillomaviruses play in the etiology of human carcinomas. 6. The complete sequencing of the BPV-1 genome.

Methods Employed: Nucleic acid hybridization, molecular cloning of DNA genomes, restriction endonuclease analysis, tissue culture, transcriptional analysis using hybridization techniques, the S1 nuclease digestion technique of Berk and Sharp, and DNA sequencing.

Major Findings: 1. We have molecularly cloned the complete genomes of the human papillomaviruses types 1a, 1b, 2, and 4, and the complete genomes of the bovine papillomaviruses types 1 and 2 in E. coli using the certified plasmid vector pBR322. Papers describing these experiments are listed below and have been published in the Journal of Virology and in the Cold Spring Harbor publication entitled Viruses in Naturally Cancers.

2. In collaboration with Dr. Lowy of the Dermatology Branch, we have assayed the biologic activity of the cloned papillomavirus DNAs and found that the cloned BPV-1 and BPV-2 DNAs are each capable of transforming mouse C127 cells, and mouse NIH 3T3 cells in tissue culture. In addition, we have found that a specific subgenomic fragment of the bovine papillomavirus is also capable of inducing these tranformed foci. In contrast, under well-controlled experiments we have been unable to demonstrate a transforming capability in this system using any of the cloned human papillomavirus DNAs which we have established.

3. Dr. Law has established that mouse cells transformed by the bovine papillomavirus, the cloned bovine papillomavirus DNA, or the subgenomic transforming region, all contain circular, extrachromosomal viral DNA molecules. In a reconstruction experiment, he has shown the sensitivity of the blotting system that he used was such that it would have detected as little as .1-.2 copies per diploid genome if it had been integrated within the mouse host cell DNA. From this we concluded that integration of the viral DNA is not required for the initiation or maintenance of the transformed state by the bovine papillomavirus. This is in marked contrast to other known viral transformation systems in which integration of the viral DNA is associated with transformation. A paper describing the state of the viral DNA in papillomavirus transformed mouse cells is in press in PNAS.

4. Dr. Heilman has been mapping the viral transcripts present in bovine papillomavirus transformed cells. The major transcript appears not to be spliced and maps from approximately .34 to .45 map units on the BPV-1 genome.

In addition two minor transcripts can be detected on the S1 gels and she is currently evaluating their map positions. In addition, she has analyzed the messenger RNA which can be purified from bovine fibropapillomas and finds that there are additional transcripts present in the fibropapillomas which are not present in the transformed cells and is currently mapping these messenger RNAs.

Publications

1. Lowy, D.R., Dvoretzky, I., Shober, R., Law, M.-F., Engel, L.W., and Howley, P.M.: In vitro tumorigenic transformation by a defined sub-genomic fragment of bovine papilloma virus DNA. Nature 287: 72-74, 1980.

2. Howley, P.M., Law, M.-F., Heilman, C.A., Engel, L.W., Alonso, M.C., Lancaster, W.D., Israel, M.A., and Lowy, D.R.: Molecular characterization of papillomavirus genomes. In Essex, M., Todaro, G., and zur Hausen, H. (Eds.): Viruses in Naturally Occurring Cancers. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory, 1980, pp. 233-247.

3. Heilman, C.A., Law, M.-F., Israel, M.A., and Howley, P.M.: Cloning of human papillomavirus genomic DNAs and analysis of homologous polynucleotide sequences. J. Virol. 36: 395-407, 1980.

4. Law, M.-F., Lowy, D.R., Dvoretzky, I., and Howley, P.M.: Mouse cells transformed by bovine papillomavirus contain only episomal viral DNA sequences. PNAS, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00547-01 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Use of Papillomavirus DNAs as Eukaryotic Cloning Vectors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Howley	Senior Surgeon	LP	NCI
OTHER:	N. Sarver	ACS Fellow	LP	NCI
	M.-F. Law	Expert	LP	NCI
	G. Khoury	Medical Director	LMV	NCI
	P. Gruss	Visiting Scientist	LMV	NCI

COOPERATING UNITS (if any)

Laboratory of Molecular Virology, NCI

LAB/BRANCH
Laboratory of Pathology

SECTION
Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The papillomaviruses are a group of viruses which have never been successfully propagated in tissue culture. Recently the molecular cloning of the viral genomes of selected papillomaviruses has permitted studies on their molecular biology. The bovine papillomaviruses are capable of transforming certain mouse fibroblast lines as well as certain rat fibroblast lines. The viral DNA in these transformed lines is maintained exclusively as extrachromosomal molecules. The extrachromosomal nature of the viral DNA in these lines together with the selected malignant phenotype suggested that the papillomaviruses may be a useful eukaryotic cloning vector. We have cloned the rat preproinsulin gene together with the transforming region of the bovine papillomavirus and assessed the ability of the papillomaviruses to be used as cloning vectors.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: 1. To establish whether papillomaviruses can be used as effective eukaryotic cloning vectors. 2. To segregate the transformation capability of the bovine papillomavirus from the extrachromosomal replication function of the bovine papillomavirus in assessing its use as a eukaryotic cloning vector. 3. To link to a transformation negative, replication positive segment of the bovine papillomavirus a dominant selective marker (the E. coli HGPRT gene) and assess its ability as a eukaryotic cloning vector. 4. To assess the ability of the human papillomavirus genomes to replicate autonomously in murine as well as primate and human cells when linked to a dominant selective marker such as the E. coli HGPRT gene. 5. To determine the capacity of the bovine papillomavirus to carry an exogenous gene without integrating into the host chromosome. 6. Assess the ability of this cloning system to deliver a gene to a cell in such a manner as to affect site specific integration.

Methods Employed: Nucleic acid hybridization, restriction endonuclease analysis, immunologic techniques, bacterial cloning, tissue culture, DNA sequencing, and DNA transfection.

Major Findings: We have demonstrated the utility of the bovine papillomavirus transforming segment as a eukaryotic cloning vector. We have hooked the 69% transforming segment of the bovine papillomavirus type 1 to the 1.62 kilobase segment of the rat preproinsulin gene and cloned this hybrid DNA into pBR322. After cleaving the DNA away from the prokaryotic sequences, we have taken the hybrid BPV T_{69}-$_rI_1$ DNA and used it to transfect C127 mouse cells. We have selected 48 independent clones and shown that each of these contains both the bovine papillomavirus DNA and the rat preproinsulin DNA. By blot hybridization analysis of one of these lines (NS-8), we have shown that the BPV DNA is covalently linked to the rat preproinsulin DNA and is maintained predominantly as an extrachromosomal circular molecule. By S1 exonuclease and Exo VII exonuclease analysis, we have shown that the authentic messenger RNA for rat preproinsulin is made within these cells. By immunoprecipitation and specific competition using unlabeled proinsulin, we have shown that these transformed lines selected for their malignant transformed phenotype produce an authentic rat proinsulin molecule. This molecule is secreted into the media by all 48 lines. Manuscripts describing these experiments are currently in press and are listed below.

Dr. Law has recently cloned the E. coli HGPRT gene (arranged in a modified SV40 early transcriptional unit as originally constructed by Richard Mulligan and Paul Berg) into a recombinant molecule containing the transforming region of the bovine papillomavirus in pBR322. Dr. Law in collaboration with Bruce Howard of the Laboratory of Molecular Biology, National Cancer Institute, have used this DNA to transform susceptible mouse cells and select the transformants either for their malignant phenotype or for their ability to survive in a selective media containing mycophenolic acid (MPA). Dr. Law has shown that mouse cells selected for their malignant transformed phenotype are able to grow in selective media and in addition, the mouse cells selected for their ability to grow in this otherwise poison media are demonstrating a malignant

phenotype. Current experiments are underway to establish the physical state of the DNA within these transformed cells.

Publications

1. Sarver, N., Gruss, P., Law, M.-F., Khoury, G., and Howley, P.M.: Bovine papillomavirus DNA - a novel eukaryotic cloning vector. Molec. Cell. Biol., in press.

2. Sarver, N., Gruss, P., Law, M.-F., Khoury, G., and Howley, P.M.: Rat insulin gene covalently linked to bovine papillomavirus DNA as expressed in transformed mouse cells. In Brown, D. and Fox, C.R. (Eds.): ICN-UCLA Symposia on Molecular and Cellular Biology. Vol. XXIII, Developmental Biology Using Purified Genes, Academic Press, New York, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00546-01 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Specific Incorporation of a Fluorescent Probe by Transformed Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L.M. Neckers	Expert	LP	NCI
	J. Cossman	IPA (Senior Investigator)	LP	NCI
	E.S. Jaffe	Chief, Hematopathology Section	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The extrinsic fluorescent probe, merocyanine 540 (MC540), has been reported to be specifically taken up by leukemic lymphocytes. We are investigating the potential use of this compound as a marker for transformed lymphocytes in spleen and lymph nodes. By using a fluorescence-activated cell sorter (FACS), we are attempting to isolate the stained cells for further microscopic study. Using dual fluorescent labeling techniques, we will also determine whether all subsets of transformed lymphocytes react with MC540, or whether MC540 uptake is limited to a population of these cells.

Finally, we feel that the degree of MC540 staining may be related to lymphoma subtype. Preliminary data suggest that nodular lymphomas stain weakest, followed by the mixed cell type, with lymphoblastic staining the strongest. We are currently extending these observations.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To determine the usefulness of MC540 staining as a marker for transformed lymphocytes.

Methods Employed: Cells are stained with the dye and/or monoclonal antibodies to surface markers. These cells are then analyzed and/or sorted with a FACS.

Major Findings: To date, we have seen that MC540 stains many transformed lymphocyte cell lines, as well as lymphocytes isolated from spleen and lymph nodes. Normal PBL and cells from uninvolved nodes and spleens are very lightly stained.

Significance to Biomedical Research and the Program of the Institute: Some evidence exists that MC540 can be used to stain transformed, but not normal lymphocytes. If this is the case, use of the dye would be a rapid, simple way to quantify and isolate malignant cells from a mixed population for further study.

Proposed Course: Staining of more individual cases are necessary before any conclusions can be drawn. More detailed investigations on the staining of lymphocyte subtypes will also be undertaken.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00548-01 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Monoaminergic Receptors on Lymphocytes Visualized by FACS Analysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L.M. Neckers	Expert	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several centrally acting biogenic amines have been shown to bind specifically and with high affinity to lymphocyte receptors, followed by alteration of lymphocyte function. We are developing fluorescent amine derivatives with which to study these receptors in vivo using a fluorescence-activated cell sorter (FACS). We will characterize the presence of these receptors on lymphocyte subsets defined by monoclonal antibodies and determine whether their presence on these subsets has any functional significance. Long term studies to measure these receptors in transformed lymphocytes will also be undertaken.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To develop techniques for studying biogenic amine receptors on lymphocytes by FACS analysis; to classify these receptors as to lymphocyte subtype.

Methods Employed: Fluorescent derivitization of biogenic amines followed by FACS analysis.

Major Findings: To date, we have demonstrated the feasibility of measuring histamine receptors on lymphocytes with these techniques.

Significance to Biomedical Research and the Program of the Institute: Although various lymphocyte receptors for biogenic amines have been found, *in vivo* measurement of these receptors has not been possible. Visualization and subtyping of these receptors will add to our understanding of their function in both normal and abnormal immunological processes.

Proposed Course: To continue developing fluorescent probes for biogenic amine receptors on lymphocytes and using them for FACS analysis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00550-01 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunologic Characterization of Malignant Lymphomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.S. Jaffe	Chief, Hematopathology Section	LP	NCI
OTHER:	J. Cossman	IPA (Senior Investigator)	LP	NCI
	R.I. Fisher	Senior Investigator	M	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to assess the clinical and pathologic significance of the immunologic characterization of human malignant lymphomas, fresh biopsy tissues are obtained from patients referred to the Clinical Center for treatment. Biopsies are obtained with patient permission prior to therapy and processed in the Hematopathology Section. The neoplastic cells are characterized as to their origin from T cells, B cells, or histiocytes, and can in addition be identified as belonging to specific developmental and functional subpopulations. This data is then correlated with clinical and pathologic data.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To determine the clinical importance of immunologic phenotype, and determine if it correlates with clinical presentation, stage, response to therapy or survival. To determine the correlation of immunologic phenotype with conventional morphology.

Methods Employed: Analysis of a wide variety of immunologic, cytochemical and biochemical markers including the identification of the following: complement receptors (CR1 and CR2), receptors for the Fc fragment of IgG and IgM, sheep erythrocyte receptors, surface immunoglobulins for individual heavy and light chains, terminal deoxynucleotidyl transferase, acid phosphate, tartrate resistant acid phosphatase, alpha-napthyl butyrate esterase, acid alpha-napthyl acetate esterase, alkaline phosphatase, beta-glucuronidase.

Major Findings: Diffuse aggressive non-Hodgkin's lymphomas are immunologically heterogeneous. Surface immunotype cannot be predicted by conventional morphology. Lymphomas of peripheral T lymphocytic origin have some unique clinical and pathologic features, in part due to the production of lymphokines by the neoplastic cells.

Significance to Biomedical Research and the Program of the Institute: This information will affect future development of clinical protocols, as tumors of differing immunotypes may require different therapies. This has already been shown to be true for lymphoblastic lymphoma. Neoplastic expansions often permit the identification of normal cellular phenotypes not previously recognized, and lead to increased understanding of the immune system.

Publications

1. Jaffe, E.S., Strauchen, J.A., and Berard, C.W.: Predictability of immunologic phenotype by an immunohistologic classification in large cell lymphomas. Am. J. Clin. Pathol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00551-01 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Stimulation of Phagocytosis by a Peripheral T-cell Lymphoma Derived Lymphokine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.R. Simrell	Medical Officer (Research)	LP	NCI
OTHER:	E.S. Jaffe	Chief, Hematopathology Section	LP	NCI
	J. Cossman	IPA (Senior Investigator)	LP	NCI
	L.M. Neckers	Expert	LP	NCI
	G. Crabtree	IPA	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Certain patients with malignant lymphomas originating from peripheral T-cells develop a rapidly fatal syndrome which mimics malignant histiocytosis. It is suspected that the pathogenetic mechanism of this phenomenon may involve a lymphokine produced by the neoplastic T-cell which can stimulate the phagocytic cells of the reticuloendothelial system. In order to test this hypothesis, neoplastic cells from fresh biopsies of patients with malignant lymphoma are placed in overnight culture, and supernatants are tested for the presence of soluble factors which are able to affect human phagocytic cells in vitro.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To determine whether certain human malignant lymphomas (especially those of peripheral T-cell origin) produce a factor (or factors) which can stimulate the phagocytic ability of human macrophages.

Methods Employed: Neoplastic cells are placed in tissue culture and 24 hour supernatants tested for their ability to induce an increase in the number of Fc receptors and to enhance the phagocytic activity of the human promyelocytic cell line HL60, the macrophage-like cell line U937, and normal peripheral blood monocytes. Fc receptors are assayed by measuring the specific Fc receptor dependent binding of ^{125}I-IgG. Phagocytosis is assayed using IgG coated OX-RBC or 1.5 u fluorescent beads.

Major Findings: Preliminary results suggest that occasional peripheral T-cell lymphomas may secrete a factor which stimulates the phagocytosis of antibody coated RBC but not beads. This effect may be mediated by an increase in the number of Fc receptors on the macrophage cell surface. Normal peripheral blood lymphocytes stimulated in an allogeneic mixed leucocyte reaction or by CON-A elaborate a factor which induces Fc receptors, phagocytosis and adherence of macrophages.

Significance to Biomedical Research and the Program of the Institute: Some patients with peripheral T-cell lymphomas develop a syndrome resembling malignant histiocytosis characterized by fever, hepatosplenomegaly, and pancytopenia associated with histiocytosis and marked erythrophagocytosis within the reticuloendothelial system. The demonstration that neoplastic T-cells from such a patient can secrete a factor capable of stimulating macrophage in vitro gives some insight into the pathogenesis of this syndrome, and also contributes to a greater understanding of the nature of normal lymphocyte/macrophage interaction.

Proposed Course: Continued screening of malignant lymphoma patients to accumulate more cases of factor producing peripheral T-cell lymphoma, attempts to establish a cell line from these cases, preliminary characterization of the factor(s) involved.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00552-01 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Malignant Lymphomas: Analysis with Monoclonal Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Cossman	IPA (Senior Investigator)	LP	NCI
OTHER:	L.M. Neckers	Expert	LP	NCI
	E.S. Jaffe	Chief, Hematopathology Section	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A variety of monoclonal antibodies (hybridomas) have been recently developed that distinguish among classes of normal human lymphocytes and identify discrete stages of differentiation. In addition new monoclonal antibodies against human B cells are being developed in our laboratory. We are using a battery of these antibodies to determine the phenotypes of human malignant lymphomas using a Fluorescence Activated Cell Sorter (FACS-II). The phenotypic expression of these neoplastic lymphocytes is then related to normal lymphocytes and is useful in diagnosis and monitoring of patients' tumors during therapy.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To identify and characterize human malignant lymphomas.

Methods Employed: Immunofluorescence FACS and computer analysis; tissue culture; hybridoma development.

Major Findings: 1. Intra-tumor phenotype heterogeneity suggestive of potential selective problems during therapy. 2. A large class of peripheral T cell lymphomas previously considered rare. 3. Documentation of reported specificity of monoclonal antibodies.

Significance to Biomedical Research and the Program of the Institute: Monoclonal antibodies have great potential for both diagnosis and treatment of malignant lymphomas. We have the best characterized series of monoclonal antibody phenotyping of lymphoma in existence. This information is invaluable to our understanding of the biology of neoplastic lymphocytes, for diagnosis, for conventional therapy and for future monoclonal antibody therapy.

Proposed Course: 1. Determine phenotypes of malignant lymphomas using monoclonal antibody. 2. Produce specific anti-human B cell monoclonal antibodies. 3. Produce antibodies specific for primitive differentiation antigens.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00553-01 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Control of Fibrinogen Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.R. Crabtree	IPA	LP	NCI
	J.A. Kant	Expert	LP	NCI

COOPERATING UNITS (if any)

Laboratory of Biochemistry, NCI

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying the control of fibrinogen gene expression as a model for investigating the mechanisms of coordinate gene expression. We have found the levels of fibrinogen mRNA rapidly increase after defibrinating rats with Malayan pit viper venom. During this response each of the three fibrinogen mRNA's increase at the same time, rate and to nearly the same extent; thus their expression is highly coordinated. We have constructed cDNA and genomic clones for each of the fibrinogen genes and are analyzing structural similarities between the genes which might account for their coordinate regulation. We have also began cloning the human fibrinogen genes and plan to study the human afibrinogenemias as a model of the effects of mutation on gene expression.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: 1) To develop a model system to study the factors controlling and coordinating the expression of genes during differentiation and development. 2) To understand the molecular genetics of the human dysfibrinogenemias and afibrinogenemias.

Methods Employed: cDNA was prepared from purified mRNA and cloned in pBR322. Fibrinogen clones were identified by hybrid-selection and translation. Human and rat genomic libraries were screened, and the clones mapped by Southern blotting, R-looping and heteroduplex mapping.

Major Findings: We have found that mRNA levels for fibrinogen increase 10-38 fold following defibrination. This response may be mediated by a feedback mechanism involving fibrin split products. We have mapped the genes for the α and ′ chains of fibrinogen and find they are tightly linked and that there are two genes for the ′ chain.

Significance to Biomedical Research and the Program of the Institute: The most recent evidence indicates that malignancy may be caused by inappropriate expression of normal cellular genes. If this proves correct, it will be essential to understand the factors controlling gene expression to permit specific therapeutic intervention. We hope that the system we have developed will be useful in understanding the general mechanism controlling gene expression. Secondly, understanding the control of fibrinogen synthesis may allow more effective treatment of thrombotic disease.

Proposed Course: In the future we hope to look for homologous regulatory regions in or about the fibrinogen genes and explore the mutations which result in the human afibrinogenemias.

Publications

1. Crabtree, G.R., and Kant, J.A.: Molecular cloning of cDNA for a family of coordinately regulated genes; the α, β, and ′ chains of fibrinogen. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00517-40 LP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Report from the Pathological Technology Section

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: B.J. Coolidge — Chief, Path. Tech. (Supv. Bio. Lab. Tech. (Micro)) — LP — NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Pathological Technology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	0	5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Stained tissue sections are the fundamental basis of all clinical and experimental studies of cancer. The Section prepares histological sections for the investigators of the National Cancer Institute. It makes available all the established routine and special stains and in addition develops and provides the current experimental methods of tissue preparation such as enzyme stains and specific histological stains.

PHS-6040
(Rev. 2-81)

October 1, 1980 - September 30, 1981

Number of investigators	76
Number of pieces of tissue	18,320
Number of bottles of tissue	9,234
Number of blocks cut	11,301
Number of blocks cut serially	1,138
Number of frozen blocks cut	188
Number of blocks recut	2,286
Number of slides stained H & E	47,359
Number of slides stained special	6,045
Number of unstained slides	13,478
Number of slides, H & E, special and unstained	66,882

SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY REPORT
DCBD, NCI
October 1, 1980 through September 1, 1981

The Dermatology Branch conducts both clinical and basic research studying the etiology, diagnosis and treatment of inflammatory and malignant diseases involving the skin and the host's response to these diseases. The basic research involves biochemical as well as biological studies of skin and is subdivided into eight separate, though frequently interacting, areas. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center (approximately 1500 patients are seen in consultation each year). The main research achievements of the Dermatology Branch for the past year are as follows:

Therapy of Skin Cancer and Disorders of Keratinization:

We are continuing to evaluate safety and effectiveness of new oral and topical agents particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne. Oral 13-cis-retinoic acid was effective in the treatment of skin cancer, and a variety of disorders of keratinization (lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris), and cystic acne. An oral synthetic aromatic derivative of retinoic acid (RO-10-9359) was similarly tested and found to be more effective and less toxic than 13-cis-retinoic acid in the treatment of the disorders of keratinization. A topical synthetic aromatic retinoid (RO-11-1430) was tested in a double-blind manner against placebo cream and found to be ineffective in the treatment of disorders of keratinization. A high initial, low maintenance dosage of 13-cis-retinoic acid was found to be a comparably effective schedule but less productive of toxicity than previously used continuous high-dosage schedules in the treatment of cystic acne. With regard to safety, we are continuing to closely monitor long term and short term side effects of the retinoids. In the past year we completed a prospective analysis of patients treated with 13-cis-retinoic acid which showed small but significant elevations in plasma lipids and changes in lipoproteins. The changes returned to normal after therapy was stopped. Patients treated with RO-10-9359 had similar changes which were dose dependent and responsive to dietary management.

The profound beneficial effect of 13-cis-retinoic acid in the treatment of acne and both retinoids in the treatment of cutaneous keratinizing diseases indicates that other keratinizing disorders of man, for instance, preneoplastic squamous metaplasia of tracheo-bronchial and urinary bladder epithelial origin, could be successfully treated with the synthetic retinoids. Treatment of the keratinizing dermatoses and acne may also provide useful information in the evaluation of newer and potentially more potent and less toxic synthetic retinoids.

In conjunction with these clinical studies we have evaluated the morphologic and biochemical effects of Vitamin A and its analogs on skin. Topical all-trans retinoic acid, but not systemic 13-cis-retinoic acid, increased gap junction density and decreased desmosome density in treated basal cell carcinomas. This indicates that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms. In addition, a specific cytosol retinol binding protein has been identified in mouse skin and human skin from patients with Darier's disease, psoriasis and basal cell carcinomas.

Studies of DNA Repair in Normal Human Cells and in Cells from Patients with Xeroderma Pigmentosum and Neurodegenerative Disorders:

UV-radiation is believed to be the major cause of the most common type of human cancer, cancer of the skin. Patients with xeroderma pigmentosum (XP) are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor development in XP patients would, therefore, elucidate the role of DNA repair in preventing, in normal humans, those cancers which may be due to certain chemical and physical carcinogens. Extension of this work to include study of other diseases in which DNA repair may be defective, especially those associated with an unusually high incidence of cancer such as ataxia telangiectasia, will further increase our understanding of the relationships between DNA repair processes and carcinogenesis. XP patients' sun-exposed skin ages much more rapidly than normal humans skin. Some XP patients develop neurological abnormalities which are due to the early death of neurons. Our studies of the DNA repair defects are providing an understanding of the relationship of DNA repair processes and abnormal aging. Our most recent studies have been conducted with cells from patients with various degenerative neurological diseases who have neurological lesions which are similar to those seen in some XP patients and in patients with ataxia telangiectasia. The studies are designed to elucidate the pathogenesis of these disorders as well as to develop presymptomatic diagnostic tests. We assess the the biological effectiveness of DNA repair primarily by *in vitro* assays of cell survival after treatment of the cells with DNA damaging agents. We have used the trypan blue exclusion test to determine the number of lymphoblasts surviving after irradiation with x-rays. Under conditions of maximum sensitivity of the assay, that is, when ataxia telangiectasia homozygote and heterozygote lines could be distinguished from normal lines, we detected hypersensitivity to x-rays in lymphocyte lines from patients with the following diseases: Huntington disease, familial dysautonomia, olivopentocerebellar atrophy, tuberous sclerosis, muscular dystrophy and retinitis pigmentosa. Lymphoblast lines from 11 other inherited diseases tested were not hypersensitive to x-rays. This post x-ray survival assay with

lymphocyte lines has currently lost its sensitivity and can no longer distinguish any line from normal except the ataxia telangiectasia homozygote lines. Studies have been underway in an attempt to determine the cause of the loss of sensitivity of this assay.

With Dr. Dominic A. A. Scudiero we have detected hypersensitivity to the lethal effects of N-methy-N-nitrosoguanidine (MNNG), a mutagenic and carcinogenic DNA-damaging chemical, in fibroblasts from patients with the following disorders: Huntington disease, infantile spinal muscular atrophy, spinocerebellar ataxia familial dysautonomia, tuberous sclerosis, muscular dystrophy and retinitis pigmentosa. This hypersensitivity may provide the basis for a prenatal diagnostic test for these diseases in tissues from fetuses at risk for these disorders. Elucidation of the molecular basis for the hypersensitivity may shed light on why most of these disorders, while hypersensitive to the lethal effects of MNNG, are not characterized by an abnormally increased incidence of cancers. Such studies may also determine whether the hypersensitivity reflects inherited defects in DNA repair mechanisms.

In addition, we have initiated cytogenetic studies in order to determine whether DNA-damaging agents induce abnormal numbers of chromosome abnormalities in cells from patients with primary neuronal degeneration. The development of such a cytogenetic test would make it possible to detect hypersensitivity to DNA-damaging agents within a few days, as opposed to the currently employed colony-forming test which requires two to three weeks.

Immunopathologic Mechanisms Involved in Inflammatory and Neoplastic Skin Diseases:

During the past year we have continued our studies of the immunopathology of skin diseases in two major areas. The first is in cell mediated immunity and the role of epidermal Langerhans cells in the induction and expression of cell mediated immune responses. We have demonstrated, using a simultaneous rosetting and immunofluorescence technique and X irradiated bone marrow reconstituted mice, that epidermal Langerhans cells are derived from precursor cells in the bone marrow. The cell turnover is very slow as only one third of the cells are replaced in the first month after chimerization. These Langerhans cells have been shown to be critical for antigen presentation *in vitro* as well as *in vivo*. Our technique for sensitizing mice involves using haptenated epidermal cell suspensions and injecting the cells by various routes. When the haptenated epidermal cells are injected intraperitoneally, there is strong and long lasting contact sensitivity induced. However, if other cell types (i.e. spleen) are haptenated and injected intraperitoneally, a state of specific immunologic tolerance ensues. If the epidermal cell suspensions are depleted of Langerhans cells or if an epidermal cell line (devoid of Langerhans cells) are haptenated and injected intraperitoneally, tolerance ensues as well. Thus the Langerhans cells have been shown to be critical for the induction of allergic contact hypersensitivity. As ultraviolet light has been shown to affect the rejection of UV induced tumors we have also explored the effects of UV light on the ability of Langerhans cells to induce sensitization. We have shown that, although UV has no effect on surface marker characteristics of Langerhans cells (i.e. Ia antigens and Fc receptors), UV light irradiation of epidermal cells and their subsequent haptenation and injection into syngeneic mice abrogates their ability to sensitize. Indeed, these UV

irradiated Langerhans cells induce specific suppressor T cells which are responsible for the hyporesponsiveness which results. In addition, and in collaboration with Dr. J. Oppenheim (NIDR) we have shown that epidermal cells, devoid of Langerhans cells, and epidermal cell cultures produce factors with Interleukin 1-like activity. This factor has been termed ETAF (epidrmal cell derived thymocyte activating factor). The biological functions and biochemical characteristics are currently under study.

The second major area of study is the identification and characterization of antigens and antibodies involved in the pathophysiology of some of the so-called autoimmune skin diseases. We are currently concentrating on cell surface antigens involved in the disease called pemphigus and on the basement membrane zone constituents involved in normal physiology and in the disease called pemphigoid. In this latter regard we have utilized cell culture, immunoprecipitation and fluorographic techniques to identify the pemphigoid antigen which is a glycoprotein with a molecular weight of approximately 220K. This antigen is clearly distinct from laminin and other basement proteins. Interestingly, patients with this disease have antibodies with specifics directed against the same antigen. With regard to cell surface antigens, we are currently producing monoclonal antibodies with specificity for squamous cell surfaces. We are also currently attempting to identify the pemphigus antigen and to study its role in cell adhesion.

Biochemical Characterization of Mammalian Melanosomes:

We are continuing our studies of the abnormal proteins produced in malignant melanoma. These studies are aimed at elucidating the mechanism of formation of these atypical proteins, as well as their importance to the immunology of melanoma and possible immunotherapy potential. The melanosome in normal mouse tissue is composed of multiple proteins, many of which are loosely bound and easily extracted. These probably constitute the proteins of the limiting membrane of the organelle. Another protein is of lower molecular weight and is tightly bound to the granule, and probably constitutes the structural, fibrillar protein; it appears to be the protein which complexes with the melanin polymer. Melanosomal proteins from melanoma tissues vary in structure from those of normal tissue. Several of the proteins seem to be missing completely from these granules, which are also structurally distinguishable from normal granules by electron microscopy. Perhaps more importantly, many of the proteins in malanoma melanosomes are unique and are not found in normal melanin granules. This has been found to be the case in human melanoma as well. In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules, resolved a slight, but significant, difference of isoelectric points (4.8 and 4.5, respectively). There also is a difference of 10,000 MW between the two proteins; both have amino acid contents which are identical with respect to 13 amino acids, but differ significantly with regards to four amino acids (asp, glu, val, arg). The carboxy and amino terminals however are identical. Peptide mapping has revealed that amino acid sequences are deleted in 3 or more regions of the abnormal protein. Other proteins in these tissues seem to differ in a similar manner. It has been found that tumor-specific proteins similar to these can be found in the serologic fluids of melanoma patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro.

The observations concerning the aberrant biochemical characteristics of the melanoma melanosome indicate that the control of melanogenesis in malignant melanoma is in some manner affected by carcinogenesis. These results indicate that although melanogenesis takes place, its metabolic pattern is abnormal. There are a wide range of implications of this research for possible immunotherapy and/or immunoassay of human malignant melanosomes.

We are also studying the role of tyrosinase in melanogenesis in normal tissues and the importance of the altered enzyme found in melanoma. Since this enzyme is essential for melanin biosynthesis, it is a unique system for the study of enzymatic control mechanisms in normal and malignant tissues. We have examined the status of enzymatic control of melanogenesis and have found that tyrosinase is the enzyme responsible for melanin synthesis in both murine and avian pigmentary systems. It has been found in this study that tyrosinase is under allosteric control and that phospholipids may play a part in the expression of the enzyme's activity. It has also been found that this allosteric regulation in murine melanomas is altered; this perhaps explains the atypical melanosome formation in these tissues. Recent evidence has been found which supports the theory that the enzyme is additionally controlled by enzyme-associated factors which can either inhibit or stimulate the production of pigment. It is hoped that further study of the cause of enzymatic differences in the malignant tissues will provide insights into the nature of neoplastic transformation.

Tumor Virus Expression In Vitro and In Vivo:

Papilloma viruses are a common cause of epidermal tumors in man. Some lesions induced by these viruses may undergo malignant conversion. Virtually nothing had been known about the functional organization of the genomes of these viruses nor how lesions progress from a benign to a malignant state. The definition of a transforming region for bovine papilloma virus DNA represents a first step towards understanding how these tumors are formed and change. Using our recently described *in vitro* transformation assay for the bovine papilloma virus and for its molecularly cloned genomic DNA we found that cells transformed by virions, which contain circular viral DNA, or those transformed by linear viral DNA molecules contain multiple unintegrated circular viral DNA copies. No integrated viral DNA has been detected, although 0.5 copies per cell could easily have been detected. The results indicate that the linear cloned viral DNA has recircularized and that the maintenance of viral induced transformation is probably mediated via the unintegrated viral DNA molecules.

In our studies of Harvey murine sarcoma virus (Ha-MuSV), we last year localized the transforming region to the 5' half of the viral DNA. This year, we localized the 1 kb which encode the viral $_{p}21$ (*src*) transforming protein to a segment near the 5' end of the viral DNA. Efficient transformation by this segment was achieved only if the viral long terminal repeat (LTR) was ligated upstream from the $_{p}21$ coding region. Using sequences from the $_{p}21$ coding region as a probe, sequences related to this region have been found to be highly conserved evolutionarily, since cells from avian and mammalian species, including man, contain one or more copies of homologous sequences. This viral *src* probe has also been used to molecularly clone two $_{p}21$ cell homologs from normal rat DNA, the species from which Ha-MuSV originated. Both genes contain about 1 kb which are homologous to Ha-MuSV DNA, in the $_{p}21$ coding region. In

one gene, the homologous sequences are colinear with Ha-MuSV while the second gene contains intervening sequences between the regions of homology. Ligation of the viral LTR upstream from either cell gene enables either cell gene to induce transformation of NIH3T3 cells. The transformed cells express high $_{p}21$ levels, while untransformed cells express low $_{p}21$ levels. The $_{p}21$ produced by the gene without intervening sequences is identical to the Ha-MuSV $_{p}21$, while the $_{p}21$ from the other gene appears to be more closely related to the $_{p}21$ produced by normal cells. These results suggest that the level of $_{p}21$ can control the growth cells and that the $_{p}21$ coding region of Ha-MuSV was derived from the gene without intervening sequences.

Applications of Scanning Electron Microscopy to Soft Biological Tissues:

Our goals with the scanning electron microscope (SEM) have been to develop new methods and interpretive criteria for the study of a wide variety of soft biological tissues, and thereby capitalize on the unique capabilities of this instrument in providing 1) improved survey and sampling as an adjunct to other studies, 2) an unparalleled three-dimensional view of cell surface phenomena, and 3) an unusual potential for cytochemical studies.

In the past year we continued our studies of the influence of cell cycle on cultured cell topography. We are examining HeLa cell monolayers grown in unperturbed cultures and parallel samples harvested by mitotic shakeoff and incubated to synchronize cells in the G1, S, and G2 stages of the cell cycle. The cells are exposed to tritiated thymidine for a brief period and fixed. After Feulgen staining, LM photomicrographs are taken of all cells in systematically selected areas of each sample. The samples are then dried and coated with evaporated carbon, and these same cells are micrographed by SEM. The samples are next dipped in liquid emulsion and processed as autoradiographs (ARG), whereupon the same cells are again examined and micrographed either by LM or by SEM in the backscattered electron (BSE) imaging mode. Individual cells are scored as to experiment number, type of specimen (unperturbed culture, synchronization category), stage in the cell cycle (phase of mitosis, labelled or unlabelled interphase), and several morphologic parameters (height and incidence of microvilli, blebs and ruffles), and these scores compiled in WYLBUR for data processing. This information will assist all investigators examining the topography of unsynchronized monolayer cells to account for the influence of cell cycle.

Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments:

One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. We have continued our studies of the keratin filaments and of a basic protein called "filaggrin", which are the most prominent intracellular components of all epidermal cells. A study of their chemistry, structure and biosynthesis in both normal and abnormal epidermis will be of profound importance in studying tumors in this tissue.

We have shown that a histidine-rich protein isolated from rat epidermis specifically aggregates epidermal keratin filaments from several species *in vitro* to form a highly-ordered fiber. Electron microscopy of such fibers

reveals a pattern of filaments 70-80 A in diameter embedded in a darker-staining background, or matrix. This structure is typical of the "keratin pattern" seen in the fully-differentiated stratum corneum of the epidermis. This suggests strongly that the basic protein is the matrix protein of epidermis. Our work constitutes the first real evidence for and demonstration of the role of a matrix protein in the epidermis. The interaction between the basic protein and filaments is highly specific since other fibrous proteins do not form the ordered structures. Therefore, there are structural features unique to keratin filaments which recognize the basic protein. One practical limitation of such studies has been the difficulty in isolation of the basic protein. In an effort to resolve this, we have developed a very simple method for isolating large quantities of the similar protein from mouse epidermal stratum corneum which functions in the same way as the rat protein. Its chemical and functional properties will now be studied in detail. Studies on the interaction between the basic protein and defined fragments of filaments and filament subunits are underway to characterize the nature and specificity of the associations between these two components in the epidermis. Interestingly, in certain diseases of the epidermis involving abnormal keratinization, such as psoriasis, the amount of the basic protein is greatly diminished from normal. There may be a relationship between the absence of the basic protein and presence of abnormal keratin filaments which could provide important information on the disease itself. Since this basic protein also aggregates the intermediate filaments from all sources so far examined we have chosen for it a new functionally-specific name, filaggrin.

In other studies we (in collaboration with Dr. M.M. Gottesman-LMB,DCBD and Dr. R.D. Goldman-Dept Anatomy, Northwestern University) have shown that the 10nm filaments of a variety of epithelial and mesenchymal cell-types grown in culture are morphologically very similar to epidermal keratin filaments, and interestingly, also possess a three-chain unit that is structurally identical that of keratin filaments. Partial specific cleavage of purified 10 nm filament subunits and bovine epidermal keratin subunits indicate that the subunits are all distinctly different, but they are structurally very similar. Such studies have permitted the construction of subunit domain maps that show two regions of α-helix of M_r 13,000 on each subunit, interspersed by regions of a non α-helix that vary in size between different subunits. Most of these studies have been done with the 10nm filaments of BHK-21 and CHO cells since these are readily available in large quantities, but similar comparative work is also planned or underway with the filaments of HeLa cells, and the neurofilaments isolated from cattle brain and squid giant axons. The single protein decamin and desmin, the principal intermediate filament subunits of fibroblasts and muscle cells, respectively, are capable of filament assembly by themselves in vitro; that is, they form homopolymer filaments. All keratin filaments, in contrast certain at least two demonstrably different subunits; that is they are obligate copolymer filaments. The presence of 10 nm filaments in cells is obviously extremely important and the structural studies of this type will provide insights into their function in normal and transformed cells.

Detection and Analysis of Circulating Immune Complexes:

Increasing evidence indicates that circulating antigen-antibody complexes play a role in the pathogenesis of a variety of dermatologic, rheumatologic,

neoplastic and infectious disease states. We have identified and partially characterized the immune complexes which exist in several diseases i.e. Sjogren's syndrome, mixed cryoglobulinemia, mixed connective tissue disease, acute and chronic schistosomiasis, hepatitis and various types of cutneous and systemic vasculitis, utilizing two highly sensitive radioimmunoassays for detecting immune complexes i.e. ^{125}I-C1q binding assay and the Raji cell radioimmunoassay. We have recently developed a new, sensitive radioimmunoassay for the detection of IgA containing immune complexes and have demonstrated that patients with dermatitis herpetiformis, gluten-sensitive enteropathy and IgA nephropathy, among others, have circulating IgA containing immune complexes. We have examined the correlation between absolute levels of circulating immune complexes, the extent and severity of clinical disease, and reticuloendothelial system function. We have also examined the influence that certain genes of the major histocompatibility complex exert on immune function in vivo and in vitro in humans. In this regard we have demonstrated an Fc receptor reticuloendothelial system clearance defect in 50% of patients with dermatitis herpetiformis and have found that a high percentage of HLA-B8/DRw3 positive normal individuals also have delayed splenic clearance of IgG coated autologous erythrocytes indicating abnormal FcIgG receptor function of splenic macrophages. In attempts to determine the extent of this FcIgG receptor defect we studied lymphocyte receptors. We found that normal HLA-B8/DRw3 positive individuals and HLA-B8/DRw3 positive dermatitis herpetiformis patients also have decreased numbers peripheral blood lymphocytes bearing receptors for the Fc portion of IgG. These HLA-B8/DRw3 patients and controls were also shown to have increased numbers of spontaneous immunoglobulin secreting cells in their peripheral blood as measured by a plaque forming assay.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03657-07 D

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.I. Katz	Senior Investigator	Derm NCI
Other:	M. Iijima	Visiting Fellow	Derm NCI
	R. Hall	Clinical Associate	Derm NCI
	J. Stanley	IPA	Derm NCI
	D. Sauder	Visiting Associate	Derm NCI

COOPERATING UNITS (if any)

Metabolism Branch, Immunology Branch, NCI, LCI, NIAID, NIDR

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	4.5	1.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Pemphigus, herpes gestationis, bullous pemphigoid and dermatitis herpetiformis are blistering skin diseases all of which are associated with so-called auto-immune phenomena. We have identified and characterized serum and in vivo bound antibodies in these diseases. Our efforts have centered around the mechanisms of tissue destruction, ultrastructure, and ultra-structural localization of antibodies. Immunogenetic considerations with regard to HLA associations and the identification of specific B-lymphocyte antigens have provided tools to study the triggering mechanisms involved in the early stages of an abnormal immune response. Another major effort is the study of Langerhans cells in inflammatory and immunologically mediated reactions. We have identified the origin of this cell which probably represents the peripheral most limb of the immune response in the skin and have identified its immunological function both in vivo and in vitro. Identification and functional characterization of alloantigens on epidermal cells and of basement membrane constituents are other integral parts of the overall program. We have identified a unique basement membrane zone antigen in human skin, the bullous pemphigoid antigen, and are currently producing monoclonal antibodies to human skin components.

Project Description

Objectives:

1) To investigate the mechanisms involved in the expression of certain immunologic skin diseases, namely pemphigus, bullous pemphigoid, dermatitis herpetiformis and herpes gestationis.

2) To determine the cell surface characteristics of the lymphocytes and epidermal cells involved in these diseases.

3) To determine whether or not genetic factors are important in the developments of these diseases.

4) To determine the ultrastructure and ultrastructural localization of antibodies in blistering skin diseases.

5) To better understand the distribution of collagen types in human skin and to characterize the chemical constituents of the basement membrane zone.

6) To determine the functional capabilities of Ia-bearing epidermal cells (Langerhans cells) in mice, guinea pigs, and humans as these cells probably play an integral role in antigen presentation and in allogeneic T cell activation.

7) To identify and characterize normal cell surface, basement membrane and cytoplasmic structures in human skin and to determine their possible role in skin cancer, wound healing, and in blistering diseases.

Material:

Skin biopsies, either punch of Castroviejo keratome slices are used. Also the small intestine of patients with dermatitis herpetiformis are studied in order to determine their antigen binding characteristics. Blister fluid studies for inflammatory mediators are also under investigation. The roofs of suction blisters are also assessed for the distribution of Langerhans Cells. Guinea pigs, mice and rabbits are used for the identification of lymphocyte and epidermal cell surface antigens as well as for the production of antibodies. Mouse skin is used to prepare epidermal cell suspensions which are used for sensitization.

Methods Employed:

Direct and Indirect Immunofluorescence, Cell Mediated Cytotoxicity, Immunochemical methods for identifying immunoglobulins and immune complexes. Radioimmunoassays. Mixed leukocyte cultures and *in vitro* antigen priming studies. Delayed type hypersensitivity reactions including contact hypersensitivity are generated by skin painting and by the injection of haplenated cells into syngeneic mice. Radioimmunoprecipitation techniques are

also employed as are standard techniques for the production of monoclonal antibodies.

Major Findings:

1) Laminin, a non collagenous glycoprotein, is localized to the lamina lucida of the basement membrane. It is a widely distributed basement membrane zone protein.

2) By cell culture and immunoprecipitation techniques we have demonstrated that the bullous pemphigoid antigen, a unique glycoprotein of stratified squamous epithelia, is produced by epidermal cells and has a molecular weight of 220Kd.

3) Langerhans cells are the only epidermal cells in mice to bear Fc-IgG receptors and express Ia antigens. This had been a disputed point for many years and has finally been resolved by combined immunofluorescent and rosetting techniques.

4) Langerhans cells are derived from bone marrow precursor cells. By using the above cited techniques and bone marrow reconstituted, irradiated animals we showed that donor marrow is the source of the Langerhans cells.

5) In vivo studies indicate that Langerhans cells play an integral role in the induction of contact hypersensitivity. Epidermal cells devoid of Langerhans cells cannot perform this function.

6) Ultraviolet light irradiation affects the antigen presenting function of Langerhans cells in such a way so that when UV irradiated Langerhans cells are irradiated and then injected subcutaneously, they induce a state of specific immunological tolerance.

7) Keratinocytes devoid of Langerhans cells produce a factor which has Interleukin (IL) I activity; that is , it enhances PHA induced thymocyte proliferation. It has many of the same physicochemical characteristics of IL 1. We have termed this factor ETAF.

8) We have produced monoclonal antibodies against normal epidermal cell constituents and are currently characterizing the antigens and studying their role in various pathologic states, such as wound healing and skin tumor formation.

Significance to Cancer Research:

A basic understanding of immunologic injury in various autoimmune disease states is important in interpreting and furthering current concepts in self-recognition. Pemphigus is associated with thymoma and myasthenia gravis and its study may provide a clue as to the association between pemphigus and other malignancies. Our in depth studies of herpes gestationis, an immunologic blistering disease of pregnancy, may provide important clues to maternal-fetal interactions. Studies are underway to determine whether

there is an association between dermatitis herpetiformis, gluten sensitive enteropathy and cancer. The role of Langerhans cells in immune reactions in the skin and their possible function or dysfunction after ultraviolet light exposure should provide some insight into their role in skin tumor formation. The study of the basement membrane and its disruption by cancer cells is essential to the study of tumor invasion.

Proposed Course of Project:

Outlined above.

Publications:

1. Tamaki, K., Katz, S.I.: Ontogeny of Langerhans Cells. J. Invest. Derm. 75: 12-13, 1980

2. Hall, R., Lawley, T., and Katz, S.I.: Dermatitis Herpetiformis in Pseudo-Allergic Reactions. Vol. 2 (In press).

3. Stingl, G., Tamaki, K., and Katz, S.I.: Origin and Function of Epidermal Langerhans Cells. Immunological Reviews 53: 149-174, 1980.

4. Katz, S.I.: The role of Langerhans cells in immunity. Arch Derm 116: 1361, 1980.

5. Katz, S.I., Strober, W., Hall, R., Lawley, T.J.: Dermatitis herpetiformis: the skin and the gut. Annals of Internal Medicine 93: 857-874, 1980.

6. Green, I., Stingl, G., Shevach, E.M., Katz, S.I.: Antigen presentation and allogeneic stimulation by Langerhans cells. 75: 44-45, 1980.

7. Stanley, J.R., Hawley-Nelson, P., Poirier, M., Katz, S.I., Yuspa, S.H.: Detection of pemphigoid antigen, pemphigus antigen and keratin filaments by indirect immunofluorescence in cultured human epidermal cells. J. Invest. Derm. 75: 183-186, 1980.

8. Foidart, J.M., Yaar, M., Hall, R.P., Gaspard, U., Katz, S.I.: Immunopathological and clinical studies in herpes gestationis. Br. J. of Ob. and Gyn. 88: 153-159, 1981.

9. Hintner, H., Stingl, G., Schuler, G., Fritsch, P., Stanley, J., Katz, S., Wolff, K.: Immunofluorescence Mapping of Antigenic Determinants Within the Dermo-Epidermal Junction in Mechanobullous Diseases. J. Invest. Dermatol. 76: 113-118, 1981.

10. Yaar, M., Stanley, J.R., Katz, S.I.: Retinoic Acid Delays the Terminal Differentiation of Keratinocytes in Suspension Culture. J. Invest. Dermatol. 76: 363-366, 1981.

11. Katz, S.I., Dermatitis herpetiformis in Immunodermatology ed Safai and Good. p. 377-388, 1981.

12. Yaoita, H., Briggaman, R.A., Lawley, T.J., Provost, T.T., and Katz, S.I.: Epidermolysis Bullosa Acquisita: Ultrastructural and Immunological Studies. J. Invest. Dermatol. 76: 288-292, 1981.

13. Tamaki, K., Fujiwara, H., Katz, S.I.: The role of epidermal cells in the induction and suppression of contact sensitivity. J. Invest. Derm. 76: 275-278, 1981.

14. Stanley, J.R., Alvarez, D., Bere, E.W., Eaglstein, W.H., Katz, S.I.: Detection of basement membrane zone antigens during epidermal wound healing in pigs. J. Invest. Dermatol. (In press).

15. Tamaki, K., Fujiwara, H., Levy, R.B., Shearer, G.M., Katz, S.I.: Hapten specific TNP-reactive cytotoxic effector cells using epidermal cells as targets. J. Invest. Dermatol. (In press).

16. Stanley, J.R., Hawley-Nelson, P., Yuspa, S.H., SHevach, E.M., Katz, S.I.: Characterization of bullous pemphigoid antigen-a unique basement membrane protein of stratified squamous epithelia. Cell (In press).

17. Sauder, D.N., Tamaki, K., Moshell, A.N., Fujiwara, H., Katz, S.I.: Induction of tolerance to topically applied TNCB, using TNP-conjugated ultraviolet light irradiated epidermal cells. J. Immunol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03638-12 D

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies of DNA Repair in Human Degenerative Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J.H. Robbins	Senior Investigator	Derm NCI
A.N. Moshell	Guest Worker	Derm NCI
R.G. Scarpinato	Clinical Associate	Derm NCI
K.I. Snowden	Gnotobiotics Unit Head	VRB DRS
S. Potkay	Chief, Small Animal Unit	VRB DRS
R.E. Tarone	Mathematical Statistician	B NCI
L.R. Seguin-Spillman	Staff Fellow	Derm NCI
D.A. Scudiero	DNA Repair Lab	Chem. Carcin. Prog.,FCRC
R. Polinsky	Expert	LCS, NIMH, NIH
J.A. Barranger	Senior Investigator	DMN, NINCDS, NIH
C.D. Lytle	Research Biochemist.	
	Experimental Studies Br., Div. Biolg. Effects., FDA.	

COOPERATING UNITS (if any)

Small Animal Section, VRB, DRS: Biometry Branch, DCCP, NCI.

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.6	3.6	3.0

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Studies in this laboratory are designed to elucidate the role of DNA repair processes in human diseases and in carcinogenesis and in normal and abnormal aging. Most studies have been conducted with cells from patients with xeroderma pigmentosum (XP) who have defective DNA repair plus multiple cutaneous malignancies, premature aging of sun-exposed skin and the nervous system. Studies are currently being performed on cells from patients with degenerative neurological deseases such as Huntington's disease, ataxia telangiectasia, familial dysautonomia, olivopontocerebellar atrophy, spinal muscular atrophy, muscular dystrophy, and retinitis pigmentosa. These studies are designed to elucidate the pathogenesis of these disorders as well as to develop presymptomatic diagnostic tests. We assess the biological effectiveness of DNA repair primarily by in vitro assays of cell survival after treatment of the cells with DNA damaging agents.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

To study DNA repair processes in normal cells, cells from patients with each of the eight genetic forms of xeroderma pigmentosum (XP), and cells from patients with other diseases in which defective DNA repair is claimed or suspected, including degenerative diseases involving the somatic and/or autonomic nervous systems, skeletal muscle, and the retina; to determine the relationship between the function of such repair processes and 1) carcinogenesis, mutagenesis, cytotoxicity and 2) the clinical findings of photosensitivity, premature aging, and UV-carcinogenesis; to develop presymptomatic diagnostic tests for the aforesaid disorders.

The objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Materials:

Cells are obtained from established cell repositories, from hospitalized patients and outpatients at the NIH, the University of Minnesota Hospitals, Minneapolis, Minn. (Dr. Jonathan Wirtschafter), and the Veterans Administration Hospital, Fargo, ND (Dr. Roger Brumback). Cells currently under study are dermal fibroblasts and lymphocyte cell lines.

Methods Employed:

Fibroblast and lymphocyte lines are cultured in the absence of antibiotics in laminar flow hoods.

The survival of fibroblasts after irradiation with UV or X-rays, or after treatment with chemicals, is performed by counting the number of colonies which form. The survival of lymphocytes is determined by their ability to exclude the vital dye trypan blue. Unscheduled DNA synthesis is determined by determining autoradiographically the UV-induced incorporation of tritiated thymidine. Host-cell reactivation is determined by the formation of plaques in fibroblast monolayers by irradiated herpes simplex virus (Dr. Lytle).

Major Findings:

1. The ability of UV-irradiated lymphocyte lines to exclude the vital dye trypan blue provides a very rapid and sensitive assay of the biological effectiveness of DNA repair. By comparing the post-UV survival of XP lymphocyte lines with the post-UV CFA of the patients' fibroblast strains, we have defined the experimental parameters of the post-UV lymphoblast trypan blue viability test.

2. We have used the trypan blue exclusion test to determine the number of lymphoblasts surviving after irradiation with x-rays. Under conditions of maximum sensitivity of the assay, that is, when ataxia telangiectasia homozygote and heterozygote lines could be distinguished from normal lines, we detected hypersensitivity to x-rays in lymphocyte lines from patients with the following diseases: Huntington disease, familial dysantonomia, olivopontocerebellar atrophy, tuberous sclerosis, muscular dystrophy and retinitis pigmentosa. Lymphoblast lines from 11 other inherited diseases tested were not hypersensitive to x-rays. This post x-ray survival assay with lymphocyte lines has currently lost its sensitivity and can no longer distinguish any line from normal except the ataxia telangiectasia homozygote lines. Studies have been underway in an attempt to determine the cause of the loss of sensitivity of this assay.

3. With Dr. Dominic A. Scudiero we have detected hypersensitivity to the lethal effects of N-methy-N'-nitro-N-nitrosoguanidine (MNNG), a mutagenic and carcinogenic DNA-damaging chemical, in fibroblasts from patients with the following disorders: Huntington disease, infantile spinal muscular atrophy, spinocerebellar ataxia, familial dysautonomia tuberous sclerosis, muscular dystrophy and retinitis pigmentosa. This hypersensitivity may provide the basis for a prenatal diagnostic test for these diseases in tissues from fetuses at risk for these disorders. Elucidation of the molecular basis for the hypersensitivity may shed light on why most of these disorders, while hypersensitive to the lethal effects of MNNG, are not characterized by an abnormally increased incidence of cancers. Such studies may also determine whether the hypersensitivity reflects inherited defects in DNA repair mechanisms.

4. With Dr. David Lytle we have studied the host-cell reactivation capacity of fibroblasts from patients with the aforesaid neurodegenerations. Their host-cell reactivation of x-rayed or UV-irradiated adenovirus was in the normal range. Thus, these diseases have no defect in their repair of the damaged virus.

5. Cytogenetic studies are being conducted to determine if DNA-damaging agents induce abnormal numbers of chromosome abnormalities in cells from patients with primary neuronal degeneration. The development of such a cytogenetic test would make it possible to detect hypersensitivity to DNA-damaging agents within a few days, as opposed to the currently employed colony-forming test which requires two to three weeks.

6. We have determined the post x-ray colony-forming ability of fibroblasts from patients with neurological disease. Our results generally parallel those of other investigators. One of two tuberous sclerosis strains was hypersensitive. The two Huntington disease strains were in the low normal range. Thus, since these strains were all hypersensitive to MNNG, the post x-ray survival test is shown to be considerably less useful than the MNNG test.

7. We have studied cells from a patient who has both Cockayne syndrome and xeroderma pigmentosum. This combination of disease has been seen in only one previous patient. The patient's lymphocyte line was hypersensitive to the lethal effects of UV radiation. His fibroblasts had a decreased rate of UV induced unscheduled synthesis. With Dr. Lytle we have shown this patients' fibroblasts to have impaired host-cell reactivation of UV damaged DNA. The occurrence in this patient of these two rare diseases with DNA repair defects (Cockayne syndrome and xeroderma pigmentosum) establishes an association between these diseases.

8. In collaboration with Dr. Polinsky we are obtaining skin biopsies and blood samples from clinically well-characterized patients with premature death of nerve cells. In collaboration with Dr. Barranger we are obtaining skin and blood biopsies from patients with tuberous sclerosis.

9. Arrangements have been made to establish at the Institute for Medical Research, Camden, NJ, lymphocyte lines and fibroblast strains from patients with degenerative neurological diseases. Over 180 strains and lines have been established, making it possible for investigators to study these diseases in tissue culture.

10. Attempts were made to accomplish in vitro malignant transformation of XP and normal fibroblasts by UV light. The irradiated cells were injected into nude mice to determine if malignant tumors would form. The Small Animal Section has kept the nude mice alive and well following their injection with irradiated human cells. No tumors have developed in these mice from the injected cells.

Significance to Biomedical Research and the Program of the Institute:

UV-radiation and ionizing radiation are causes of human cancer. Patients with XP are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor development in XP patients would, therefore, elucidate the role of DNA repair in preventing in normal humans those cancers which may be due to certain chemical and physical carcinogens. Extension of this work to include study of diseases

in which DNA repair may be defective, such as those with hypersensitivity to ionizing radiation, will further increase our understanding of the relationships between DNA repair processes and carcinogenesis. XP patients' sun-exposed skin ages much more rapidly than normal humans' skin. Some XP patients develop neurological abnormalities that are due to the premature death of neurons. Our studies of the DNA repair defects are providing an understanding of the relationship of DNA repair processes to these aging phenomena in XP. Thus, our study of aging in XP organs is providing important knowledge as to how properly functioning DNA repair processes prevent such aging in normal humans. Our studies of other diseases with premature death of nerve cells are also providing an understanding of such abnormal aging phenomena and of the relationship between ionizing radiation-type damage to DNA carcinogenesis.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Publications:

1. Moshell, A.N., Tarone, R.E., Newfield, S.A., Andrews, A.D. and Robbins, J.H.: A simple and rapid method for evaluating the survival of xeroderma pigmentosum lymphoid lines after irradiation with ultraviolet light. In Vitro (In press), 1981.

2. Barrett, S.F., Tarone, R.E., Moshell, A.N., Ganges, M.B., and Robbins, J.H.: The post-UV colony-forming ability of normal fibroblast strains and of the xeroderma pigmentosum group G strain. J. Invest. Dermatol. 76: 59-62, 1981.

3. Kraemer, K.H. and Robbins, J.H.: Xerodema Pigmentosum, in Current Therapy in Dermatology, Stuart Maddin, Editor, W.B. Saunders Co., Phil. (In press), 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03663-05 D

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Tumor virus expression in vitro and in vivo

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.R. Lowy	Senior Investigator	Derm NCI
	E. Chang	Expert	Derm NCI
	I. Dvoretzky	Visiting Associate	Derm NCI
	Y. Nakabayashi	Visiting Fellow	Derm NCI

COOPERATING UNITS (if any)
1. Laboratory of Tumor Virus Genetics, NCI
2. Pediatric Oncology Branch, NCI
3. Laboratory of Pathology, NCI

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	4.0	1.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Using our recently described in vitro transformation assay for the bovine papilloma virus and for its molecularly cloned genomic DNA, mouse cells transformed either by virus or by viral DNA have been shown to contain multiple unintegrated viral DNA copies in the absence of detectable integrated viral DNA. Efficient transformation by the p21 (src) coding region of Harvey murine sarcoma virus (Ha-MuSV) DNA requires that the viral long terminal repeat (LTR) be ligated to the p21 coding region. Two independent p21 coding genes have been cloned from normal rat cell DNA; one gene is colinear with the viral p21 coding region, while the second gene contains intervening sequences. When the Ha-MuSV LTR is ligated to either gene, it can transform NIH3T3 cells, associated with high intracellular p21 levels. The orgin and formation of mink cytopathic focus-forming (MCF) murine leukemia viruses (MuLV) has also been studied. Endogenous MCF-like viral DNAs have have been found in AKR and other mouse strains. The entire env of some non-pathogenic MCF viruses has apparently been derived from these sequences. In spontaneous murine thymic tumors, one or more of these MCF-like sequences have regularly recombined with a specific region of the 3' end of ecotropic env. Pathogenic MCF viruses also contain these ecotropic virus-derived recombinant sequences.

Project Description

Objectives:

1. To evaluate the effects of hormones and other chemicals on endogenous and exogenous virus expression.

2. To gain insight into how the state of differentiation of a cell regulates virus expression.

3. To study viral recombination, especially as it relates to the development of sarcoma viruses and leukemia viruses.

4. To improve the biologic assay of DNA-mediated gene transfer (transfection).

5. To determine the effect of a variety of physical and chemical treatments on the biological activity of specific DNAs (viral and cellular).

6. To determine the physical differences between unexpressed and expressed endogenous DNA, since transfection of expressed viral DNA is more easily accomplished than that of unexpressed viral DNA.

7. To investigate the mechanism of activation of endogenous C-type viruses by halogenated pyrimidines.

8. To define the portions of sarcoma virus genomes which induce cellular transformation.

9. To study the structure and biological potential of gene sequences from normal cells which are homologous to the transforming region of sarcoma viruses.

10. To study the cellular origin of retrovirus components.

11. To analyze the normal function of genes which encode the transforming function of sarcoma viruses.

12. To screen human tumors for their high expression of gene sequences homologous to the transforming genes of sarcoma viruses and for the ability of their DNA to induce cell transformation.

13. To develop a radioimmune assay for wart viral protein(s).

14. To develop nucleic acid hybridization procedures for wart viral DNA.

15. To propagate and study wart viruses in tissue culture.

16. To measure the relatedness of viruses of warts from different patients,

incluing those from patients with epidermodysplasia verruciformis (EDV), laryngeal papillomas, condyloma accuminata, flat warts, common warts, plantar warts, and warts associated with immunodeficiency.

17. To look for wart specific nucleic acid and/or antigen in skin cancers of EDV, kerato-acanthomas, and other tumors.

18. Relatedness of wart viruses to other papova viruses.

Methods Employed:

1. Treatment of cells or animals with hormones, other chemicals, and tumor viruses.

2. Detection of retrovirus expression biologically by XC plaque test and focus induction.

3. Radioimmune assays for viral antigens.

4. For the isolation of proviral genomes, DNA is extracted from tissue culture cells or tumors by the Marmur technique, except that proteinase K is used instead of pronase, since higher molecular weight DNA is obtained by this modification.

5. For isolation of unintegrated viral DNA, it is purified by the Hirt procedure.

6. Transfection of DNA utilizes the calcium phosphate technique of Graham and Van der Eb as modified by Stow and Wilkie.

7. Virus is grown in sensitive tissue culture cells and purified by sucrose density centrifugation in a zonal rotor. Isotopically labeled single stranded viral DNA probes are synthesized in an endogenous reverse transcriptase reaction carried out in the presence of antinomycin D or in exogenous reactions following purification of viral RNA on sucrose density gradients.

8. Nucleic acid hybridization between specific probes and cell or viral nucleic acids is carried out in liquid and analyzed by S_1 nuclease. Hybridization is is also performed by transferring nucleic acids from agarose gels to nitrocellulose filters.

9. Cell or viral DNAs are assayed for biologic activity in a DNA transfection assay. This activity is then correlated with the expression of the transfected genes in the cells.

10. Unintegrated and integrated viral genomes are molecularly cloned and amplified in prokaryotic systems.

Major Findings:

1. Last year, our laboratory described an in vitro transformation system for bovine papilloma virus. Using this assay for molecularly cloned viral DNA, transformation has been induced by a fragment which contains 69% of the viral genome. The cells are non-productively transformed and do not express virion antigens.

   The cells transformed by virions, which contain circular viral DNA, or those transformed by linear viral DNA molecules contain multiple unintegrated circular viral DNA copies. No integrated viral DNA has been detected, although 0.5 copies per cell could easily have been detected. The results indicate that the linear cloned viral DNA has recircularized and that the maintenance of viral induced transformation is probably mediated via the unintegrated viral DNA molecules.

2. In our studies of Harvey murine sarcoma virus (Ha-MuSV), we last year localized the transforming region to the 5' half of the viral DNA. This year, we localized the 1 kb which encode the viral p21 (src) transforming protein to a segment near the 5' end of the viral DNA. Efficient transformation by this segment was achieved only if the viral long terminal repeat (LTR) was ligated upstream from the p21 coding region. Using sequences from the p21 coding region as a probe, sequences related to this region have been found to be high conserved evolutionarily, since cells from avian and mammalian species, including man, contain one or more copies of homologous sequences. This viral src probe has also been used to molecularly clone two p21 cell homologs from normal rat DNA, the species from which Ha-MuSV originated. Both genes contain about 1 kb which are homologous to Ha-MuSV DNA, in the p21 coding region. In one gene, the homologous sequences are colinear with Ha-MuSV while the second gene contains intervening sequences between the regions of homology. Ligation of the viral LTR upstream from either cell gene enables either cell gene to induce transformation of NIH3T3 cells. The transformed cells express high p21 levels, while untransformed cells express low p21 levels. The p21 produced by the gene without intervening sequences is identical to the Ha-MuSV p21, while the p21 from the other gene appears to be more closely related to the p21 produced by normal cells. These results suggest that the level of p21 can control the growth of cells and that the p21 coding region of Ha-MuSV was derived from the gene without intervening sequences.

3. Biochemical studies of murine leukemia virus (MuLV) DNA genomes have given new insights into the relationship among these viruses and the origin and role of mink cell focus-forming (MCF) viruses. The structure of the viral DNAs from eleven MuLVs isolated from wild mice have been analyzed. The restriction endonuclease map of the U_3 region of the LTR of California mice differs significantly from that of Asian mice. The U_3 region of Asian mice is similar to that found in endogenous MuLV of inbred laboratory mice. The physical maps of amphotropic and ecotropic MuLV isolated from California mice differ primarily in their gp70 coding regions, which correlates with

biological data suggesting that the gp70 encodes the viral host range function.

The physical maps of viral DNAs of xenotropic, ecotropic, and MCF viruses isolated from AKR and other mice have been compared. As noted by others, MCF viruses have features of ecotropic and xenotropic viral DNAs. However, some differences with xenotropic viral DNAs and the apparent substitution of the entire env with non-ecotropic sequences in some MCF viruses suggested that the MCF host range might be encoded by an endogenous MCF-like viral genome. Using a viral probe specific for MCF and xenotropic env, multiple copies of apparently intact MCF-like env sequences unlinked to ecotropic env sequences have been found in cell DNA of AKR and other mouse strains. In non-pathogenic MCF viruses, these sequences have apparently been acquired intact. In pathogenic MCF, a specific region of the ecotropic genome has been retained When cell DNA from spontaneous AKR thymomas were examined, each tumor was found to contain the MCF env sequences linked to the same 3' ecotropic sequences noted in the pathogenic MCF viruses. All tumor tissue contained this recombination, while non-tumor tissue did not. The tumors were clonal in origin and did not contain novel ecotropic viral genomes. The results suggest that specific recombination between ecotropic virus and endogenous MCF env sequences are important in the pathogenesis of AKR tumors.

Significance to Cancer Research:

Papilloma viruses are a common cause of epidermal tumors in man. Some lesions induced by these viruses may undergo malignant conversion. Very little is known about the functional organization of the genomes of these viruses or how lesions progress from a benign to a malignant state. The definition of a transforming region for BPV DNA represents a first step towards understanding how these tumors are formed. Our results indicate that the transforming function is limited to a specific region of the viral genome and that transformation can occur independently of viral replication. The finding that the transformed cells do not contain integrated viral DNA suggests that episomal DNA can maintain cellular transformation. It seems likely that similar mechanisms may operate in human papilloma viruses. If therapies can be devised which are selective against episomal DNA, this might provide an approach for specific-antiviral therapy. Further studies will attempt to define any viral gene product(s) associated with transformation and to relate the bovine system to human papilloma viruses. This approach may lead directly to improved specific therapy for papilloma virus infection.

C-type retroviruses have been implicated in tumors of a wide variety of species, including man. The murine viruses of this group represent the best studied mammalian retroviruses; therefore they are an excellent system for the study of retrovirus induced tumorigenesis. Because retroviruses are composed of endogenous gene sequences, understanding how their expression leads to oncogenic transformation may be relevant to non-virus induced tumorigenesis as well. The cellular control of gene expression is also a fundamental problem of tumor cell

biology. Information in this area will be critical to understand how eukaryotic genes are at different times unexpressed, induced, or constitutively expressed, and will bear directly on the approaches which will be undertaken in the future to prevent or treat malignant disease of viral or non-viral origin. Our studies of Ha-MuSV indicated the the sequences in the src region are evolutionarily conserved and are present in human cells. The finding that levels of the normal endogenous p21 genes can control the growth of cells suggests that other src genes may also function in this manner. It is likely that the rat p21 gene without introns is not expressed in normal cells while the p21 gene with introns is expressed in normal cells. This suggests that cell growth may be controlled either by genes normally expressed at low levels or by some normally unexpresssed genes. These studies provide a direct experimental basis to search for the expression of src genes in spontaneous human tumors. The finding of specific recombination in the AKR tumors suggest that recombinational events between specific DNAs may be important in the pathogenesis of tumors, whether through the formation of a hybrid gene product or by increasing levels of specific genes.

Proposed Course:

Achieve stated goals.

Publications:

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10) Rands, E., Lowy, D.R., Lander, M.R., and Chattopadhyay, S.K.: Restriction endonuclease mapping of ecotropic murine leukemia viral DNAs: size and sequence heterogeneity of the long terminal repeat. Virology 108: 445-452, 1981.

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12) Law, M.-F., Lowy, D.R., Dvoretzky, I., and Howley, P.M.: Mouse cells transformed by bovine papillomavirus contain only episonal viral DNA sequences. Proc. Natl. Acad. Sci. USA, (in press).

13) Chattopadhyay, S.K., Lander, M.R., Gupta, S., Rands, E., and Lowy, D.R.: Origin of mink cytopathic focus-forming (MCF) viruses: comparison with ecotropic and xeno-tropic murine leukemia virus genomes. Virology, (in press).

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15) Ellis, R.W., DeFeo, D., Shih, T.Y., Gonda, M.A., Young, H.A., Tsucida, N., Lowy, D.R., and Sconick, E.M.: The p21 src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03659-07 D

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Therapy of Skin Cancer, Disorders of Keratinization, and Cystic Acne

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	E.G. Gross	Expert Consultant, Dermatopathologist	Derm NCI
	J.J. DiGiovanna	IPA, Univ. of Miami	Derm NCI
	D. Butkus	Visiting Fellow	Derm NCI
	G. Gantt	Registered Nurse	Derm NCI
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4.3	4.3	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oral 13-cis-retinoic acid was effective in the treatment of skin cancer, and a variety of disorders of keratinization (lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris), and cystic acne. An oral synthetic aromatic derivative of retinoic acid (RO-10-9359) was similarly tested and found to be more effective and less toxic than 13-cis-retinoic acid in the treatment of the disorders of keratinization. A topical synthetic aromatic retinoid (RO-11-1430) was tested in a double-blind manner against placebo cream and found to be ineffective in the treatment of disorders of keratinization. A high initial, low maintenance dosage of 13-cis-retinoic acid was found to be a comparably effective schedule but less productive of toxicity than previously used continuous high-dosage schedules in the treatment of cystic acne.

Prospective analysis of patients treated with 13-cis-retinoic acid showed small but significant elevations in plasma lipids and changes in lipoproteins. These changes returned to normal after therapy was stopped. Patients treated with RO-10-9359 had similar changes which were dose dependent and responsive to dietary management.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

To evaluate safety and effectiveness of new oral and topical agents particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne.

Agents:

1) 13-cis retinoic acid, 10, 20 and 40 mg capsules
2) An ethyl ester of a trimethylmethoxyphenyl derivative of retinoic acid (RO-10-9359) 10, 25 and 50 mg capsules.
3) An ethylamide of a tremethylmethoxyphenyl derivative of retinoic acid (RO-11-1430) in 0.1% cream and 0.3% gel.

Materials:

1) Keratinizing Dermatoses

   A Ichthyosis
      a) lamellar ichthyosis
      b) X-linked ichthyosis
      c) ichthyosis vulgaris
      d) epidermolytic hyperkeratosis
      e) non-bullous congenital ichthyosiform erythroderma
   B Darier's disease
   C Psoriasis
   D Keratoderma palmaris et plantaris
   E Pityriasis rubra pilaris
   F Nevus comedonicus
   G Erythrokeratodermia variabilis
   H Netherton's syndrome
   I Hailey-Hailey disease
   J Lichen planus
   K Pachyonychia congenita
   L Dyskeratosis congenita

2) Basal cell carcinoma

   A Nevoid basal cell carcinoma syndrome
   B Sunlight induced basal cell carcinomas
   C Arsenical induced basal cell carcinomas
   D X-ray induced carcinomas

3) Cystic and Conglobate acne and hidradenitis suppuritiva

Methods Employed:

1) Disorders of Keratinization

a) Oral 13-cis retinoic acid was given for 4 month courses of therapy at 1 mg/kg/day. The dose was gradually increased (up to 8 mg/kg/day) to tolerence.

b) Oral RO-10-9359 was also given for 4 month courses of therapy beginning at 0.5 mg/kg/day. The dose was increased up to 1.5 mg/kg day or to tolerence.

c) Topical RO-11-1430 cream, 0.1%, was given in a double-blind manner versus placebo to 9 patients with disorders of keratinization. Therapy was given twice daily for 4 to 8 weeks without occlusion.

2) Basal Cell Carcinoma

Oral 13-cis retinoic acid was given for 4 month courses of therapy at 1 mg/kg/day. The dose was gradually increased (up to 8 mg/kg/day) to tolerance.

3) Cystic acne

a) Patients, 16 years and older, with at least 10 cystic lesions were treated with oral 13-cis-retinoic acid in an initial pilot study (14 patients) or in a double-blind, randomized study against placebo (33 patients) or in a third study testing a high initial and low maintenance dosage schedule (40 patients).

b) In the pilot study Oral 13-cis retinoic acid was given for 4 month courses of therapy beginning at 1 mg/kg/day. The dose was gradually increased to tolerance.

c) In the double-blind designed protocol, treatment was begun at a dosage of 0.5 mg/kg/day and was only increased if there was a significant worsening of the disease.

d) In the "high-low" study, 20 patients with predominantly facial acne, they were given a high initial dose of 1.0 mg/kg/day for either 2 weeks or 4 weeks and then were given a low maintenance dose of 0.25 mg/kg/day for the remainder of a 16 week treatment period. Similarly, 20 patients with predominantly trunk acne received a high initial dose of 2.0 mg/kg/day for either 2 or 4 weeks followed by a low maintenance dose of 0.5 mg/kg/day for the remainder of the 16 week course of therapy.

Major Findings:

1. 14 patients with treatment-resistant cystic and conglobate acne was treated for 4 months with oral 13-cis retinoic acid, a synthetic isomer of naturally occurring all-trans-retinoic acid. The average maximum dose received was 2.0 mg/kg/day. 13 patients experienced complete clearing of their disease; the other had 89% improvement, as determined by the number of acne nodules and cysts present before and after therapy. Prolonged remissions, currently lasting an average of 46 months after discontinuation of therapy, have been observed in all 14 patients. One patient developed 2 lesions 12 months after discontinuation of therapy. Therapy was resumed in his case and

he is once again free of disease. No other patient has required or been given additional therapy. The mechanism of action of 13-cis-RA in the therapy of acne probably involves a direct inhibitory effect of the drug on the sebaceous gland. Evidence for this inhibitory effect comes from 3 skin biopsies in size of the sebaceous gland, and from forehead skin surface lipid film analyses, which indicated sebaceous gland inhibition by significantly lower levels of squalene and wax esters during therapy. Additionally, there was an average 84% decrease in mean forehead sebum production as compared to pre-treatment values. There was a complete return to pre-treatment values after discontinuation of therapy in the sebum composition studies but only a partial return to normal of sebum production.

2. A study comparing 13-cis-RA at initial dose of 0.5 mg/kg/day versus placebo in a double-blind format in the treatment of cystic and conglobate acne has been completed. 17 patients who initially received placebo worsened to the point where the double-blind code was broken and treatment with 13-cis-RA was begun. There was an overall 57% increase in the number of cystic lesions in this group. 16 of these 17 patients then received 13-cis-RA with a resultant 97% improvement. An additional 17 patients who had been randomly assigned to receive initial therapy with 13-cis-RA had an overall 96% reduction in number of nodules and cysts. There were an average of 46 nodules and cysts before treatment, and 2 afterwards. The average maximum dose of 13-cis-RA received by all 33 patients was 1.2 mg/kg/day, or 90 mg/day. 22 of the 33 patients have become completely free of lesions and only 3 patients have more than 3 lesions remaining. Patients who responded rapidly to a low dose were predominantly female, with facial lesions, and with an average of 27 cysts pretreatment. Slower responders requiring higher doses were predominantly male, with chest and back lesions, and with an average of 67 cysts pretreatment. 21 patients received one 4-month course of therapy; 12 received 2 courses after a 2-month treatment-free interval. 4 patients who had cleared completely after one course of therapy with 13-cis-RA had mild relapses after 4 to 12 months post-discontinuation. 3 of these 4 patients had received only 0.5 mg/kg/day during their initial course of therapy, and all cleared completely with their second course. All other patients continue to have prolonged remissions ranging from 31 to 38 months, average of 34. These results indicate that the beneficial therapeutic response is not a placebo effect, that 0.5 mg/kg/day is an effective dose particularly for facial lesions and is also productive of the common side effects. Higher doses are frequently required for control of chest and back lesions. Continued healing after discontinuation of therapy indicates that lower doses or alternate dosage schedules may also be effective.

3. At the first post-treatment follow-up visit: 1.0 mg/kg/day for 2 weeks group there was an overall 70% reduction in cysts with an 85% reduction of facial cysts, in particular. In the 1.0 mg/kg/day for 4 weeks group there was an overall 82% improvement with an 89% response on the face. In the 2.0 mg/kg/day for 2 weeks group there was an overall 86% improvement with a 96% improvement on the face, 81% on chest, 83% on the back. In the 2.0 mg/kg/

day for 4 weeks group there was an over all 77% improvement with 95% improvement on the face, 77% on the chest, and 63% on the back. Both 1.0 mg/kg/day groups had temporary, slight increases in numbers of observed cysts during the first 2 weeks of therapy, whereas the 2.0 mg/kg/day groups did not. We conclude that for most patients, especially for facial lesions, 2.0 mg/kg/day for 2 weeks is the optimal high dosage. In some patients with back acne, the high initial dose of 2.0 mg/kg/day may have to be prolonged beyond 2 or even 4 weeks. The observed toxicity during the initial high dose periods was similar to our other studies. However, once the low maintenance dose was begun there was a marked reduction initally in severity of toxicity and later in incidence. We conclude that except for a few patients with comparatively resistant back acne, the high-low dosage schedule is comparable in effectiveness to the continuous high dosage schedule of previous studies but is superior in minimizing the incidence and severity of observed toxicities.

4. 12 patients with multiple basal cell carcinomal induced by sunlight, X-ray, arsenic, or the nevoid basal cell carcinoma syndrome were treated with oral 13-cis-retinoic acid. Of 270 tumors, 43 (16%) underwent complete clinical regression. Twenty-one of 35 of these tumors when biopsied after treatment were found to be gone microscopically as well. Correlation of therapeutic response with tumor size revealed that 19 of 83 (23%) tumors 3-5 mm in diameter and 18 of 99 (18%) tumors 6 to 10 mm in diameter underwent complete clinicl regression, whereas only 6 of 88 (7%) tumors 11 mm or greater in diameter responded completely. Of the remaining tumors, 173 (64%) decreased in size and 54 (20%) were unchanged. Average maximum dosage in this group of 12 patients was 4.6 mg/kg/day with a range of 1.5 to 8.2 or 370 mg/day with a range of 120 to 660 mg/day. Duration of treatment varied from 16 to 96 weeks with an average of 56. Four patients had been sensitized and treated with DNCB six years previously. Three of these 4 patients developed a marked inflammatory response in most tumors during therapy with oral 13-cis-retinoic acid. Two of the other 8 non-DNCB exposed patients developed inflammation in a few of their tumors. However, there was no difference in the final therapeutic response between these two subgroups with 21/109 (19%) tumors completely regressing in the DNCB treated group and 22/161 (14%) in the DNCB unexposed group. Histologically, a dense small cell infiltrate was seen in tumors undergoing inflammation, suggesting that 13-cis-RA could either be enhancing a host immunologic response or be producing a direct cytotoxic effect on the tumor. However, in 7 of 17 (41%) non-inflammatory regressing tumors biopsied during treatment islands of squamous differentiation with horn pearl formation were observed with progression in some islands to complete replacement of tumor cells by the completely differentiated horn pearl. In tumors biopsied prior to treatment, only 14 of 85 (16%) showed evidence of keratinization. This data is consistent with an enhancement of epidermal differentiation by 13-cis-RA.

5. 57 patients with cutaneous disorders of keratinization were treated with oral 13-cis-retinoic acid, an oral synthetic retinoid from Hoffman-La Roche, Inc., Nutley, New Jersey. Diseases included Darier's disease

(DD) (9 patients), lamellar ichthyosis (LI) (10), psoriasis (PSOR) (9), pityriasis rubra pilaris (PRP) (5), keratoderma palmaris et plantaris (KPP) (4), epidermolytic hyperkeratosis (EHK) (4), non-bullous congenital ichthyosiform erythroderma (NBCIE) (3), x-linked ichthyosis (XLI) (3), Hailey-Hailey (HH) (2), and 1 each with a variant form of NBCIE, erythrokeratodermia variabilis (EKV), pachonychia congenita (PC), ichthyosis vulagaris (IV), Netherton's syndrome (NS), and porokeratosis (PORO). The patients ranged in age from 4 to 82 years. The dosage varied from 0.5 to 8.2 mg/kg/day, the duration of treatment varied from one week to over 5 years. The average maximum dosage was 160 mg/kg/day or 2 mg/kg/day. Treatment was initially given in 16 week courses of therapy with intervening 8 week treatment-free intervals. Now it is given in 6 month courses with 1-4 week intervals. Good or excellent responses were seen in DD (7), LI (8), NBCIE (3), PSOR (4), KPP (2), EKV (1), and IV (1). Partial responses were obsered in EHK (3), PRP (2), LI (1), DD (2), KPP (1), PC (1, variant-NBCIE (1). Patients showing minimal or no response included: PSOR (5), XLI (3), LP (2), HH (2), NS (1), EHK (1), PRP (1), KPP (1). One patient with PORO and one with LI stopped treatment within one week before a therapeutic evaluation could be made. The mechanism by which this synthetic retinoid alters these disease states is not known but may be related to the observed ability of vitamin A to affect epithelial differentiation. The observed variation in therapeutic response could be related to the presence or absence of specific retinoid binding proteins. Our results indicate that synthetic retinoids, such as 13-cis-RA may represent a potent new class of drugs in the treatment of cutaneous disorders of keratinization, several of which were previously treatment-resistant.

6. 75 patients with cutaneous disorders or keratinization were treated with RO-10-9359, an oral synthetic aromatic retinoid from Hoffman-La Roche, Inc., Nutley, New Jersey. This retinoid is an ethyl ester of a trimethyl-methoxy-phenyl derivative of retinoid acid. Diseases included DD (18), PSOR (23), PRP (10), LI (6), EHK (4), IV (3), KPP (2), variant-NBCIE, NBCIE (1), Kyrle's dis- (1), PC (1), HH (1), PORO (1), and LP (1). The average maximum dosage was 86 mg/day or 1.2 mg/kg/day. Duration of treatment varied from 1 week to over 3 years. Treatment was initially given in 16 week courses of therapy with 8 week treat-free intervals. Now it is given in 6 month courses with 1-4 week intervals. Good or excellent responses were seen in PSOR (8), DD (7), PRP (4), LI (3), IV (2), KPP (1), and EKV (1). Moderate responses included: PSOR (15), DD (11), PRP (6), LI (3), EHK (4), IV (1), KPP (1), variant-NBCIE (2), and Kyrle's (1). Minimal responses were noted in one patient with PORF, the lingual lesions of PC lesions of PC (1), and the oral lesions of LP (1). Worsening was observed in HH (1). Response to therapy was initially variable in psoriasis. 12 patients worsened for 8, 12, or even 16 weeks of therapy and then improved; 11 other patients with psoriasis improved immediately. All other patients with responsive diseases improved immediately upon beginning therapy. RO-10-9359 is clearly superior to 13-cis-retinoic acid in the treatment of psoriasis, IV, KPP, and EHK.

7. 9 patients with disorders of keratinization were treated with topical RO-11-1430 cream, 0.1%, in a double-blind manner against placebo. Diseases included PSOR (3), KPP (2), EHK (1), and variant NBCIE (1). 7 patients received

retinoid cream treated sites showed improvement indicating either a beneficial effect of the vehicle or an overall seasonal improvement in the patients disease. No patient exhibited a preferential beneficial effect of the retinoid cream. No systematic or local toxicities were observed. These results indicate that either RO-11-1430 is ineffective or that the 0.1% concentration is inadequate. Similar testing with the 0.3% gel will begin soon.

8. Common side effects in most patients treated with these retinoids were limited to the skin and mucous membranes and included cheilitis, facial dermatitis, conjunctivitis, xerosis, dryness of the nasal mucosa with mild nosebleeds, and easy peeling of the stratum corneum upon trauma termed "skin fragility". Aside from skin fragility, the above side effects were either more common or more severe during treatment with 13-cis-RA than with RO-10-9359. However, there were side effects that were present in higher incience in patients treated with RO-10-9359. These include: skin fragility, hair thinning (telogen), itching, palmar peeling, dry mouth with thirst, arthralgias, "stickiness" of the skin, and paronychia. Laboratory abnormalities during therapy are limited to elevations of the erythrocyte sedimentation rate, temporary low-grade elevations of the transaminases in approximately 10% of patients which return to normal values without discontinuation of therapy, and elevations of the alkaline phosphatase, LDH, and serum triglycerides in a few patients.

In the "High-Low" cystic acne study described in Methods employed, section 3d, and in Major Findings, section 3, analyses of serum lipids were performed. In those 20 patients initially receiving 1.0 mg/kg/day of 13-cis-RA, an increase of 20% in total plasma triglycerides and an increase of 6% in total plasma cholesterol was noted during therapy when compared to pretreatment values. In 9 of these 20 patients HDL-cholesterol was measured and found to be decreased by 12% during therapy. In 19 of the 20 patients initially receiving 2.0 mg/kg/day, the observed changes in serum lipids during therapy as compared to pretreatment values, were: 1) an increase of 24 mg/dl in HDL-cholesterol, 2) an increase of 5.4 mg/dl in VLDL-cholesterol, 3) a decrease of 3.6 mg/dl of HDL-cholesterol, 5) an increase of 25.5 mg/dl of total plasma cholesterol, and 5) an increase of 44.2 mg/dl of total plasma triglycerides. All values were significant at levels of $p < 0.01$.

A third group of 9 patients were retreated with 2.0 mg/kg/day for 6 months. Data from each of these three different treatment schedules indicated no significant change in triglyceride or cholesterol values after the initial rise from base line values noted after the first week of treatment. Therefore 2 mg/kg/day of 13-cis-retinoic acid over 6 months did not lead to significantly higher triglyceride or cholesterol levels than those observed with shorter treatment periods. All values returned to base line within 4 weeks of stopping therapy.

Similar changes in serum lipids and lipoproteins have been observed in 9 patients with psoriasis treated with oral RO-10-9359 at a maximum

dosage of 1.0 mg/kg/day. These changes returned towards normal with dietary management and with decreased dosage.

In general, these side effects are dose-dependent in incidence and severity, relieved by adjunctive bland therapies, well-tolerated by the patients, and totally reversible upon discontinuation of therapy.

Significance to Cancer Research:

a) These are exploratory studies of a new class of drugs (synthetic retinoids) in the treatment of skin cancer. Skin cancer should prove to be a valuable lesion for the screening of these agents because of tumors are observable, measurable, and therefore can be used as an objective indicator of response to therapy. Furthermore, the patients can be expected to be in good health, which would allow for long term studies.

b) The profound beneficial effect of 13-cis retinoic acid in the treatment of acne and both retinoids in the treatment of cutaneous keratinizing diseases indicates that other keratinizing disorders of man, for instance, preneoplastic squamous metaplasia of tracheo-bronchial and urinary bladder epithelial, could be successfully treated with the synthetic retinoids. Treatment of these keratinizing dermatoses and acne may also provide useful information in the evaluation of newer and potentially more potent and less toxic synthetic retinoids.

Proposed Course of Project:

1) Continued treatment of patients with acne, basal cell carcinomas, and disorders of keratinization with 13-cis retinoic acid (RO-43780).
2) Treatment of disorders of keratinization with topical RO-11-1430, 0.3%.
3) Continued treatment of disorders of keratinization with oral RO-10-9359.

Publications:

1) Peck, G.L., Gross, E.G., Butkus, D.: Comparative analysis of two retinoids in the treatment of disorders of keratinization, in Retinoids: Advances in Basic Research and Therapy. (Orfanos, C.E., Braun-Falco, O., Farber, E.M., Grupper, C., Polano, M., Schuppli, R. eds.) Springer-Verlag, Berlin-Heidelberg-New York, 1981 (In Press).

2) Peck, G.L.: Retinoids in Clinical Dermatology in Progress in Cutaneous Research (ed. R. Fleischmajer) Grune & Stratton, New York, 1981.

3) Olsen, T.G., Peck, G.L., Lovegrove, R.H.: Acute urinary tract obstruction in dyskeratosis congenita. J. Am. Acad. Dermatol. 4: 556-560, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03630-11 D

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effects of Vitamin A and Analogs on Chick, Mouse and Human Skin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.L. Peck	Senior Investigator	Derm NCI
	E.G. Gross	Expert Consultant, Dermatopathologist	Derm NCI
	P.M. Elias	Asst. Professor, Dept. of Dermatology	Univ. of California Medical Center
	J.J. DiGiovanna	IPA, Univ. of Miami	Derm NCI
	G. Chader	Acting Chief. Lab of Vision Research	NEI

COOPERATING UNITS (if any)

1. Dept. Dermatology, UCSF
2. Lab of Vision Research, NEI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project proposed to morphologically define the mechanism of action of vitamin A and its derivatives (retinoids) in altering epidermal differentiation in normal skin, and in benign and malignant lesions of skin. Topical all-trans retinoic acid, but not systemic 13-cis-retinoic acid, increased gap junction density and decreased desmosome density in treated basal cell carcinomas. This indicates that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms.

A specific cytosol retinol binding protein has been identified in mouse skin and human skin from patients with Darier's disease, psoriasis and basal cell carcinomas.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

1) To investigate the mechanisms governing epidermal cell differentiation.

2) To morphologically investigate the mechanism by which vitamin A and its derivatives alter epidermal cell differentiation in normal adult skin and in benign and malignant lesions.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Material:

1) 0.2-0.4mm thick, Castroviejo keratotome slices of normal and diseased human skin. Skin from patients with Ichthyosis and Darier's Disease (NCI-3643) has been used to date.

2) 3-4 mm punch biopsies of normal and diseased human skin both treated and untreated with synthetic retinoids (NCI-3643).

Major Findings:

1) Freeze-fracture replicas and thin sections of cell membranes of: 1) 11 basal cell cancers (BCC) treated twice daily for two weeks with topical 1.0% all-trans retinoic acid (RA); 2) 21 BCC treated for 2 to 17 weeks with oral 13-cis retinoic acid (CRA) (1.0-8.0 mg/kg/day); and 3) 17 BCC prior to retinoid treatment and/or after applications of vehicle alone. Both thin sections and replicas were examined and photographed in a single-blind fashion, and the density and size distribution of gap junctions and desmosomes were computed planimetrically. Neither RA nor CRA treatment appeared to influence hemidesmosome or microfilament populations. Structural changes in both treatment groups did not correlate with either tumor regression or inflammation. This indicates that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms.

2) Sucrose density centrifugation was used to identify specific binding of vitamin A to one fraction of newborn mouse skin cytosol. Specific binding was also found in cytosol from skin obtained from patients with Darier's Disease, psoriasis and basal cell carcinoma.

Significance to Cancer Research:

1) Since carcinogenesis is an instance of altered differentiation, studies of vitamin A effects on differentiation, may serve as an excellent

model for investigations of cellular control mechanisms which relate to carcinogenesis. The fact that carcinogenesis is influenced very markedly by vitamin A deficiency directly links research on vitamin An epithelial effects to cancer research.

2) Furthermore vitamin A is of value in the treatment of malignancy. The mechanisms of action of vitamin A in affecting differentiation may be related to its antineoplastic activity.

Proposed Course of Project:

1) Continued ultrastructural examination of normal skin and benign and malignant lesions of skin both treated and untreated with synthetic retinoids.

2) Continued study of the specific mechanism of vitamin A and retinoid binding to normal and diseased skin with emphasis on elucidating the mechanism of action of these drugs on skin.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03666-03 D

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Detection and Analysis of Circulating Immune Complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.J. Lawley	Expert	Derm NCI
Other:	R.P. Hall	Clinical Associate	Derm NCI
	S.I. Katz	Chief of Branch	Derm NCI
	J. Cason	Technician	Derm NCI

COOPERATING UNITS (if any)

LCI, NIAID, Metabolism Branch, NCI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.9	1.4	1.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Increasing evidence indicates that circulating antigen-antibody complexes play a role in the pathogenesis of a variety of dermatologic, rheumatologic, neoplastic and infectious disease states. We have identified and partially characterized the immune complexes which exist in several diseases i.e. Sjogren's syndrome, mixed cryoglobulinemia mixed connective tissue disease, acute and chronic schistosomiasis, hepatitis and various types of cutaneous and systemic vasculitis, utilizing two highly sensitive radioimmunoassays for detecting immune complexes i.e. ^{125}I-C1q binding assay and the Raji cell radioimmunoassay. We have developed a new, sensitive radioimmunoassay for the detection of IgA containing immune complexes. We have determined the antibody classes present in the immune complexes and examined the physiochemical characteristics of these complexes, as well as the reaction of these complexes with mediators of inflammation such as the complement system. We have examined the correlation between absolute levels of circulating immune complexes, the extent and severity of clinical disease, and reticuloendothelial system function. We have also examined the influence that certain genes of the major histocompatibility complex exert on immune function in vivo and in vitro in humans.

Project Description:

Objectives:

1) To further characterize circulating immune complexes in human diseases with regard to size and nature of the antigen, and subclass of antibody.

2) To develop reliable methods for specifically purifying immune complexes.

3) To continue clinical studies of immune complex diseases and to further assess the effects of therapy on levels of immune complexes and reticuloendothelial system clearance function.

4) To develop immune complex assays capable of detecting soluble IgM containing complexes.

5) To examine the relationships between immunologic function and HLA antigen expression in patients and normal volunteers.

6) To characterize cell surface markers of monocytes, lymphocytes, and lymphocyte subsets in an immunologically well characterized group of HLA-B8/DRw3 positive individuals using monoclonal antibodies and a fluorescein activated cell sorter.

Material:

Serum, red blood cells, white blood cells and skin biopsies from patients and controls will be used. Purified human Clq, Raji cells, and ^{125}I (Bolton-Hunter reagent) and immunospecific antisera are used in the immune complex assays. Purified IgG fraction of anti Rh(D) human antiserum and ^{51}Cr.

Methods Employed:

^{125}I-Clq binding assay, Raji cell IgG radioassay, Raji cell IgA radioassay, direct and indirect immunofluorescence, Fc specific reticuloendothelial system clearance assay, column chromatography, sucrose density gradient ultracentrifugation, monoclonal antibodies, fluorescein activated cell sorter.

Major Findings:

1) We have determined that a high percentage of patients with essential mixed cryoglobulinemia (MC) have large amounts of circulating immune complexes.

2) A large portion of these immune complexes in MC are noncryoprecipitable in addition to those that are cryoprecipitable.

3) The immune complexes in MC are mainly of the IgG type.

4) Partially purified immune complexes from some patients with MC contain hepatitis B surface antigen suggesting that this may be constituent antigen in the immune complexes found in this disease.

5) We have demonstrated that a high percentage of patients with Berger's disease (IgA nephropathy) have IgA containing circulating immune complexes.

6) These IgA immune complexes are more common early in the disease process and sediment as 9S-13S molecules.

7) We have demonstrated that high percentage of patients with psoriasis have IgA containing circulating immune complexes. The amount of IgA immune complexes correlates with pretreatment disease severity scores.

8) We have demonstrated an Fc receptor reticuloendothelial system clearance defect in 50% of patients with dermatitis herpetiformis.

9) We have found that a high percentage of HLA-B8/DRw3 positive normal individuals have delayed splenic clearance of IgG coated autologous, erythrocytes indicating abnormal FcIgG receptor function of splenic macrophages.

10) Normal HLA-B8/DRw3 positive individuals and HLA-B8/DRw3 positive dermatitis herpetiformis patients also have decreased numbers peripheral blood lymphocytes bearing receptors for the Fc portion of IgG.

11) These HLA-B8/DRw3 patients and controls were also shown to have increased numbers of spontaneous immunoglobulin secreting cells in their peripheral blood as measured by a plaque forming assay.

12) We found in several studies of patients with acute schistosomiasis that almost all patients had circulating immune complexes which tended to rise early in the course of disease, reaching maximal levels by 10 weeks after exposure.

13) The immune complex levels in patients with schistosomiasis were correlated with disease severity and the concentration of Schistosoma mansoni eggs in the stool.

14) We have found that approximately 50% of patients with gluten sensitive enteropathy (GSE) have IgA containing circulating immune complexes as well as IgG immune complexes.

15) The presence and level of immune complexes in GSE patients does not correlate with disease activity and serial studies during dietary gluten challenge showed no consistent changes in immune complex levels.

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1. Hall, R.P., Lawley, T.J., Heck, J.A., Katz, S.I.: IgA containing immune complexes in dermatitis herpetiformis, Henoch-Schonlein purpura, systemic lupus erythematosus and other diseases. Clin. Exp. Immunol. 40: 431-437, 1980.

2. Lawley, T.J., Gorevic, P.D., Hamburger, M.I., Franklin, E.C., and Frank, M.M.: Multiple types of immune complexes in patients with mixed cyroglobulinemia. J. Invest. Dermatol. 75: No. 4 297-301, 1980.

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4. Hiatt, R.A., Ottesen, E., Sotomajor, R., Lawley, T.J.: Serial observations of circulating immune complexes in patients with acute schistosomiasis. J. Infect. Dis. 142: 665-670 1980.

5. Lawley, T.J., Hall, R.P., Fauci, A.S., Katz, S.I., Hamburger, M.I., Frank, M.M.: Defective Fc receptor functions associated with the HLA-B8/DRw3 haplotype: Studies in patients with dermatitis herpetiformis and normal subjects. N. Engl. J. Med. 304: 185-192 1981.

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8. Hall, R.P., Stachura, E., Cason, J., Whiteside, T., Lawley, T.J.: IgA containing immune complexes in patients with IgA nephropathy (Berger's disease) (Submitted for publication).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03656-08 D

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Steinert	Visiting Scientist	Derm. NCI
	G.L. Peck	Senior Investigator	Derm. NCI
	W.W. Idler	Chemist	Derm. NCI
	M.I. Gullino	Laboratory Technician	Derm. NCI
	S.H. Yuspa	Branch Chief	Exper. Path. Br. DCCP, NCI
	M.M. Gottesman	Section Head	Laboratory of Mole. Biol. DCBD, NCI
	A.C. Steven	Visiting Scientist	Laboratory of Phys. Biol. NIAMDD
	R.D. Goldman	Professor	Dept. Antmy & Cell Biol. Northwestern Univ.
	B.A. Dale	Assistant Professor	Dept. Peri. & Med. Univ. of Wash.

COOPERATING UNITS (if any)

Experimental Pathology Branch, DCCP, NCI
Laboratory of Molecular Biology, DCBD, NCI
Laboratory of Physical Biology, NIAMDD

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.75	3.25	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The polypeptide chains which comprise the subunits of the keratin filaments of normal human, murine and bovine epidermis have been isolated and characterized. The subunits polymerize in vitro into native-type filaments. The repeating structural unit of the filaments consists of three chains aligned side-by-side with two discrete regions of supercoiled a-helix interspersed by regions of non-helix. Higher orders of filament ultrastructure are being investigated using image analysis procedures of filaments examined by transmission electron microscopic and scanning transmission electron microscopic techniques. Model structures generated from these methods will be computationally tested for compatibility with other physio-physico chemical data and amino acid sequence studies of individual filament subunits. The 10nm filaments of fibroblasts, muscle cells and neuronal tissues have been shown to be structurally similar to, but immunologically different from keratin filaments. A histidine-rich basic protein isolated from rat epidermis and the slightly different protein of mouse epidermis specifically aggregate keratin filaments and other 10nm filaments in a manner suggestive of an interfilamentous matrix component.

Project Description:

Objectives:

1) To characterize the ultrastucture of keratin filaments polymerized in vitro from the subunits of the filaments isolated form normal human, murine and bovine epidermis.

2) To characterize the polypeptide subunits of keratin filaments isolated from various benign and malignant human and mouse epidermal diseases.

3) To investigate the biosynthesis of the keratin filament polypeptide subunits in cultures of normal mouse epidermal cells and to investigate the effects of various drugs and carcinogens on the cells.

4) To investigate the nature of the highly-specific interaction between epidermal keratin filaments and a histidine-rich basic protein isolated from the epidermis.

5) To investigate the chemical, immunological and structural similarities between epidermal keratin filaments and 10nm filaments isolated from a variety of cell types, such as BHK-21, CHO, HeLa, and PtK1 cells grown in culture, and of muscle and neuronal tissues.

Major Findings and Methods Employed:

1) The polypeptide chains which comprise the subunits of the keratin filaments of normal bovine and murine epidermis have been isolated and individually characterized by standard protein chemical techniques. The unfractionated mixture of polypeptides or combinations of two of the purified polypeptides spontaneously polymerize in vitro in dilute salt solution into filaments that are uniformally 80 Å wide and up to 40 μm long. The polypeptide composition of these filaments, and their structure, based on electron microscopy and X-ray diffraction, are the same as the in situ keratin filaments. The stoichiometry of the recombination experiments reveals that the polypeptides are present in the filaments in the precise molar ratios of 1:2. This suggests that the epidermal keratin filament has a three-chained unit structure and this is supported by the X-ray diffraction analyses.

2) The X-ray diffraction and stoichiometric data implies that the filaments contain regions of three-chain coiled-coil α-helix; that is, the filament consists of a three-chain building block unit. This structural concept has been confirmed by partial proteolytic digestion of filaments and the subsequent isolation and characterization of α-helix-enriched fragments. The structural unit is about 20A wide by about 500 A long and and consists of three subunits aligned side-by-side with two coiled-coil α-helical segments each about 180 A long, interspersed by regions of non-α-helix.

3) The structure of this proposed three-chain unit will be probed further

to answer specific questions such as the polarity and orientation of the three protein chains within it. Isolated purified subunits will be cleaved at tryptophan and methionine residues and where possible, the frequents will be ordered along the sequence. Subsequently, intact filaments or preparations of three-chain units will be cross-linked bi-functional reagents, cleaved and from the array of fragments obtained, orientations of the three chains will be determined.

4. In collaboration with Dr. A.C. Steven, (Laboratory of Physical Biology, NIAMDD) attempts are underway to understand the higher orders of filament structure. Good negative-stained images of filaments can be subjected to optical diffraction analysis to obtain information of prominently repeating structural elements. Many filaments have to be computationally straightened in order to eliminate "noise" introduced by the curvilinear shape of the negatively-stained filaments. Diffraction images are then computationally averaged to identify the prominent repeats, which should then provide clues as to how the proposed three-chain units are assembled into the filament. Basic structural information on filaments is also derived from scanning transmission electron microscopy (STEM) of filaments. This technique will be performed at the N.I.H. STEM facility located in the Department of Biology of the Brookhaven National Laboratory (Dr. J. Wall, Director) in Upton, New York. This technique provides information on the mass of the filaments and their composite three-chain units in relation to length. Preliminary data suggests that (1) there are 7-11 three-chain units per unit length of about 500 A of filament; and (2) at least filaments assembled in vitro are polymorphic; that is, some filaments differ in the number of units they contain per unit length. The significance of this observation is unclear but may have enormously important implications in terms of the structure and function of the filaments in cells. In addition, STEM technology will provide information on the shape of intact filaments and of the three-chain units. It is expected that the application of this new technology to the study of keratin (and other related intermediate filaments, see item 9 below) will for the first time enable the construction of working models for the filaments. Optical diffraction and STEM analysis will be performed on (1) intact filaments from a variety of keratin sources as well as on the intermediate filaments of several cell types; (2) protofilamentous forms of these filaments, obtained by dissociation in low salt or in high concentrations of sodium citrate buffer, pH 2.6; (3) particles of intermediate size obtained during various early stages of filament assembly in vitro; and (4) native filaments obtained from various types of cells where possible to provide a direct comparison of in vitro and in vivo filament structural forms.

5. High resolution transmission electron microscopy of sections through normal murine epidermis, and the outer-root-sheath component of guinea pig hair follicles (which is contiguous to and continous with the epidermis) is being employed to study the structure of keratin filaments in intact tissue. In transverse cross-section, the filaments appear annular, that is, they have a region of reduced density in their center. Similar microscopic sections of filaments polymerized in vitro demonstrate this appearance also.

In addition, polymerized filaments negatively-stained with neutralized phosphotungstic acid display a region of enhanced electron density along centers. These observations suggest the filament is tubular in structure. Further detailed electron microscopic data as well as optical image diffraction and STEM analyses (see item 4 alone) are required to confirm this concept.

6. Comparisons of the polyacrylamide-gel electrophoretic profiles of the keratin subunits obtained from abnormal human epidermis such as Darier's disease and lamellar ichthyosis with those of normal epidermis show prominent differences in numbers and mobilities of bands. Also, the abnormal polypeptides show limited facility for polymerization in vitro. Therefore, there may be differences in the chemical structures of the proteins. Attempts are underway to identify such differences by comparisons of two-dimensional gel electrophoretic maps of the CNBr and NBS peptides of the proteins obtained from normal and abnormal epidermis. Filaments assembled in vitro from psoriatic epidermis are also abnormal, and interestingly, form 'paracrystalline' structures consisting of several filaments associated side-by-side in an apparently ordered manner. This may be due to the presence of an additional protein that perhaps functions like the basic protein (see item 9 below), or may be a feature characteristic of the filament proteins of psoriatic epidermis. Studies are underway to characterize this phenomenon further.

7. Normal mouse epidermal cells grown in monolayer culture can be made to synthesize the normal complement of keratin polypeptides. These proteins have been isolated and characterized by standard protein chemical techniques as done earlier with the bovine proteins. The reason why the mouse keratins are also being studied is that this cell culture system is currently being used for studies of in vitro carcinogens during transformation. Since the keratins are the principal synthetic products of the cells, they will be used as specific markers for the studies on carcinogenesis. To this end, a specific radioimmune assay has been developed to follow the changges occurring during carcinogenesis. Preliminary experiments have revealed marked alterations in the synthesis of the keratin proteins during treatment with carcinogens such as TPA and other growth effectors such as vitamin A.

8. The synthesis of the keratin filament subunits by the mouse epidermal cells implies the presence within the cells of the complete protein biosynthetic apparatus, and this system is therefore potentially very useful for studying control of differentiation in the epidermis. This is of importance as there are a number of diseases of the epidermis which may be attributed to disorders of the differentiative process of the tissue. Polyribosomes of the size necessary to synthesize the keratins can be isolated and attempts are underway to isolate the messenger RNA responsible for their synthesis.

9. In collaboration with Dr. M.M. Gottesman (Laboratory of Molecular Biology, DCBD) and Dr. R.D. Goldman and (Dept of Anatomy & Cell Biology, Northwestern University, Chicago, IL) it has been shown that the 10nm filaments of a variety of epithelial and mesenchymal cell-types grown in culture are morphologically very similar to epidermal keratin filaments, and interest-

ingly, also possess a three-chain unit that is structurally identical to that of keratin filaments. Partial specific cleavage of purified 10 nm filament subunits and bovine epidermal keratin subunits indicate that the subunits are all distinctly different, but they are structurally very similar. Such studies have permitted the construction of subunit domain maps that show two regions of α-helix of M_r 13,000 on each subunit, interspersed by regions of a non α-helix that vary in size between different subunits. Most of these studies have been done with the 10nm filaments of BHK-21 and CHO cells since these are readily available in large quantities, but similar comparative work is also planned or underway with the filaments of HeLa cells, and the neurofilaments isolated from cattle brain and squid giant axons. The single protein decamin and desmin, the principal intermediate filament subunits of fibroblasts and muscle cells, respectively, are capable of filament assembly by themselves in vitro; that is, they form homopolymer filaments. All keratin filaments, in contrast certain at least two demonstrably different subunits; that is they are obligate copolymer filaments. Preliminary studies suggest that certain neurofilament subunits are capable of homopolymer and/or copolymer filament assembly in vitro. Since all of these filaments are basically very similar (although subtlely different), it is perhaps not surprising that combinations from different sources also form native-type filaments in vitro. Hybrid filaments containing subunits from epidermis + fibroblasts, epidermis + smooth muscle, bovine epidermis + mouse epidermis, etc, have been formed. Such filaments are termed heterologous copolymers. A preliminary conclusion from these observations is that perhaps cells can modulate their 10nm filament composition with regard to specific functions. In support of this idea, BHK-21 cells contain two types of filament subunits, that characteristic of fibroblasts and that of muscle cells, which apparently copolymerize in situ to form a filament of properties intermediate between the two. The presence of 10 nm filaments in cells is obviously extremely important and the structural studies of this type will provide insights into their function in normal and transformed cells.

10. Intermediate filament subunits are phosphorylated in vivo by cyclic nucleotide dependent protein kinases. Presumably cells regulate the structure and/or function of the filaments in this way. Attempts to characterize this process will initially involve estimation of the amount of phosphate bound to subunits, its location and possible function in terms of filament assembly in vitro. Filament subunits of mouse and bovine epidermis, CHO and BHK-21 cells, smooth muscle and various neuronal tissues will be examined. The serine-phosphate content will be estimated by reaction of subunits with methylamine.

11. In collaboration with Dr. B.A. Dale (University of Washington, Seattle, WA.) it has been shown that a histidine-rich protein isolated from rat epidermis specifically aggregates epidermal keratin filaments from several species in vitro to form a highly-ordered fiber. Electron microscopy of such fibers reveals a pattern of filaments 70-80 A in diameter embedded in a darker-staining background, or matrix. This structure is typical of the "keratin pattern" seen in the fully-differentiated stratum corneum of the epidermis. This suggests strongly that the basic protein is the matrix protein of epidermis. Our work constitutes the first real evidence for and

demonstration of the role of a matrix protein in the epidermis. The interaction between the basic protein and filaments is highly specific since other fibrous proteins do not form the ordered structures. Therefore, there are structural features unique to keratin filaments which recognize the basic protein. One practical limitation of such studies has been the difficulty in isolation of the basic protein. In an effort to resolve this, we have developed a very simple method for isolating large quantities of the similar protein from mouse epidermal stratum corneum which functions in the same way as the rat protein. Its chemical and functional properties will now be studied in detail. Studies on the interaction between the basic protein and defined fragments of filaments and filament subunits are underway to characterize the nature and specificity of the associations between these two components in the epidermis. Interestingly, in certain diseases of the epidermis involving abnormal keratinization, such as psoriasis, the amount of the basic protein is greatly diminished from normal. There may be a relationship between the absence of the basic protein and presence of abnormal keratin filaments which could provide important information on the disease itself. Since this basic protein also aggregates the intermediate filaments from all sources so far examined we have chosen for it a new functionally-specific name, filaggrin. Presumably the filaggrin recognises structural features common to keratin and intermediate filaments. Studies are underway to determine the physiological significance of this result; for example it is not yet known whether filaggrin-like proteins are present in fibroblasts etc.

Significance to Cancer Research:

The epidermis offers a unique opportunity for the study of tumors not only because of the prevelance of tumors in this tissue but also because of its accessibility. One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. The keratin filaments and filaggrin are the most prominent intracellular components of all epidermal cells and therefore a study of their chemistry, structure and biosynthesis in both normal and abnormal epidermis will be of profound importance in studying tumors in this tissue. The production of these protein in a well defined cell culture system will facilitate studies of carcinogenesis *in vivo* and *in vitro*.

Proposed Course of Project:

Studies on the abnormal (benign and malignant) tissues will be done in collaboration with Dr. Gary Peck. The cell culture studies will be done in the Experimental Pathology Branch in collaboration with Dr. Yuspa. The biochemical, biophysical and electron microscopic studies of the structure of normal epidermal keratin filaments will be done in this laboratory. The computational image analysis studies of filaments will be done in collaboration with Dr. A.C. Steven. Collaborative efforts with Drs. R.D. Goldman, M.M. Gottesman and B.A. Dale will continue in the areas defined above.

Publications:

1. Cabral, F., Gottesman, M.M., Zimmerman, S.B., and Steinert, P.M., Intermediate filaments from Clinese Hamster Ovary cells contain a single protein: Comparison with more complex systems from baby hampster kidney and mouse epidermal cells. J. Biol. Chem. 256 1428-31 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB 03650-09 D

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Characterization of Mammalian Melanosomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. J. Hearing	Research Biologist	Derm NCI
OTHER:	J.M. Nicholson	Professor	Howard Univ.
	D. Gersten	Assoc. Professor	Georgetown Univ.
	J. Marchalonis	Professor	Medical Univ. of S. Carolina

COOPERATING UNITS (if any)

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Department of Pathology, Georgetown University, Washington, D.C.
Department of Biochemistry, Med. Univ. of S. Carolina, Charleston, S.C.

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The abnormal proteins produced in human and murine malignant melanoma in vivo and in vitro are being studied: These studies are aimed at elucidating the mechanisms of formation of these atypical proteins, as well as their importance to the immunology of melanoma and/or possible immunotherapy potential.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

To characterize the structural and enzymatic composition of membrane proteins of melanocytes throughout their development in vivo, both in normal and in abnormal pigmentary systems.

The objective of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Our research has been aimed primarily at characterizing the chemical composition of membrane proteins in murine and human pigment systems, both in the normal as well as in melanoma tissues. Tissues are excised and homogenized and subcellular fractions are then isolated and purified by means of an extensive series of differential centrifugations. Gel filtration and preparative polyacrylamide gel electrophoresis have enabled mg quantitites of the proteins under study to be isolated in a pure state. This has allowed the characterization of these proteins with regard to their amino acid content, isoelectric point, molecular weight, prosthetic group content, end terminal sequences, and cyanogen bromide maps. In addition, antibodies to these purified proteins have been produced in both rabbits and goats, and immunochemical characterization of these proteins is underway.

Major Findings:

The melanosome in the normal mouse tissue is composed of multiple proteins, many of which are loosely bound and easily extracted; these constitute the proteins of the limiting memrane of the organelle. Another protein is of lower moleular weight and is tightly bound to the granule, and probably constitutes the structural, fibrillar protein; it appears to be the protein which complexes with the melanin polymer. Melanosomal proteins from melanoma tissues vary in structure from those of normal tissue. Several of the proteins seem to be missing completely from these granules, which are also structurally distinguishable from normal granules by electron microscopy. Perhaps more importantly, many of the proteins in melanoma melanosomal membranes are unique and are not found in membranes of normal melanin granules. This has been found to be the case in human melanoma as well. In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules,

resolved a slight, but significant, difference of isoelectric points and a difference of 10,000 MW between the two proteins; both have amino acid contents which are identical with respect to 13 amino acids, but differ significantly with regards to four amino acids. The carboxy and amino terminals of these proteins are identical, while peptide mapping has revealed that amino acid sequences are deleted in 3 or more regions of the abnormal protein. Other proteins in these tissues seem to differ in a similar manner. It has been found that tumor-specific proteins similar to these can be found in the serologic fluids of melanoma patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro.

Significance to Cancer Research:

These observations concerning the aberrant biochemical characteristics of the melanoma melanosome indicate that the control of melanogenesis in malignant melanoma is in some manner affected by carcinogenesis. Melanomas are unusual among cancers since they do not result in complete dedifferentiation of the affected melanocyte, but allow this cell's specialzation, i.e. melanogenesis, to continue. These results indicate that although melanogenesis takes place, its metabolic pattern is abnormal.

There are a wide range of implications of this research for possible immunotherapy and/or immunoassay of human malignant melanoma. An analogous situation of altered proteins has been described in several other types of malignancies; thus this process of atypical formation of proteins may be common to neoplastic transformation. The similarity of these proteins suggests that the unique proteins present in the malignant melanocyte are an aberrant form of the analogous protein in the normal melanocyte, although the mechanism effecting this has not yet been elucidated.

Proposed Course of Project:

Since there are indications that the metabolism of melanogenesis in murine melanoma is aberrant, our research is ultimately intended to more fully characterize human melanomas, and attempt to determine the level on which these disorienting control mechanisms operate. It is hoped that further insight into such controls, be they at the level of replication, transcription, translation, or post-translation, will provide clues as to the level at which carcinogenic information is expressed.

Publications:

1. Nicholson, J.M., Montague, P.M. & Hearing, V.J.: SDS soluble but Triton X-100 insoluble normal and malignant melanosomal proteins. Pigment Cell (in press).

2. Hearing, V.J., Newburger, A.E., Ekel, T.E. and Montague, P.M.: Malignant melanoma-abnormal proteins synthesized in murine and human tissues. Pigment Cell (in press).

3. Gersten, D.M., Hearing, V.J., and Marchalonis, J.J.: Surface antigens of murine melanoma cells. Comparative Pathology, Proc. Symp. Membranes and Recognition (in press).

4. Gersten, D.M., Hearing, V.J., and Marchalonis, J.J.: Characterization of immunologically significant unique B16 melanoma proteins produced in vivo and in vitro. Proc. Natl. Acad. Sci. USA (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03649-09 D

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Enzymatic Control Mechanisms in Melanogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. J. Hearing	Research Biologist	Derm NCI
OTHER:	J. M. Nicholson	Professor	Howard Univ.
	John Pawelek	Asst Professor	Yale University

COOPERATING UNITS (if any)

Department of Chemistry, Howard University, Washington, D.C.
Dermatology Branch, Yale Univ. School of Medicine, New Haven, Conn.

LAB/BRANCH

Dermatology Branch

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NCI, NIH, Bethesda, Maryland 20205

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1.5	0.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has partially characterized the nature of tyrosinase, and determined the mechanismXof normal latency of the enzyme, and its subsequent activation in vivo. The role of this enzyme in melanogenesis in normal tissue and the importance of the altered enzyme found in melanoma tissues is being studied.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

To investigate the control mechanisms involved in regulating melanogenesis in a variety of normal and malignant tissues.

The objective of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Tyrosinase and other enzymatic activities are investigated both by spectrophotometric and radioactive assay methodology. Tissues are dissected, homogenized and fractionated into cellular fractions by means of density gradient centrifugation and differential centrifugaton. After solubilization with detergents, enzymes are further purified by gel filtration and preparative polyacrylamide gel electrophoresis. Samples are then incubated with the appropriate substrates and controls; the production of melanin and other reaction products can be followed spectrophotometrically, by liquid scintillation counting of the newly formed radioactive melanin or by products, and characterized by amino acid analysis.

Major Findings:

Since only one enzyme (tyrosinase) is essential for melanin biosynthesis, it is a unique system for the study of enzymatic control mechanisms in normal and malignant tissues. We have examined the status of enzymatic control of melanogenesis and have found that tyrosinase is the enzyme responsible for melanin synthesis in both murine and avian pigmentary systems. It has always been a subject of dispute how tyrosinase, which can be easly demonstrated to be present in an active configuration in the endoplasmic reticulum and Golgi apparatus, is inactivated in vivo in mammalian systems and subsequently activated once in situ in the melanosome. L-DOPA is the natural activator of the enzyme in vivo; we have tested over twenty L-DOPA analogs substituted in various positions, and determined the enzyme is extremely specific in its requirement for L-DOPA as a cofactor. It has been found in this study that tyrosinase is under allosteric control and that phospholipids may play a part in the expression of the enzyme's activity. It has also been found that this allosteric regulation in murine melanomas is altered; this perhaps explains the atypical melanosome formation in these tissues. Recent evidence has been found which supports the theory that the enzyme is additionally controlled by enzyme-associated factors which can either inhibit or stimulate the production of pigment.

Significance to Cancer Researach:

In view of the differences noted between the regulation of tyrosinase in normal and melanoma tissues, we feel that at least one of the primary differences between the melanogenic capabilities of these two tissues has been revealed. The fact that these controls are operational at the posttranslational level is informative; whether other levels of cellular control mechanisms are affected by carcinogenesis remains to be investigated. It is hoped that further study of the cause of these enzymatic differences in the malignant tissues will provide insights into the nature of neoplastic transformation.

Proposed Course of Project:

Differences in the control mechanisms over melanogenesis in normal and malignant melanoma tissues will continue to be investigated. The characteristics of enzymatic activity and control in various subcellular fractions are being studied. This project has been expanded to include characterization of similar metabolisms in normal and malignant human tissues.

Publications:

1) Hearing, V.J., Ekel, T.M. and Montague, P.M.: Mammalian tyrosinase-isozymic forms of the enzyme. Inter. J. Biochem. 13 99-103 (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03654-08 D

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Applications of SEM to Soft Biological Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Wetzel	Senior Investigator	Derm NCI
OTHER:	M.S. Crane	Visiting Fellow	LPD NIAID
	J.A. Dvorak	Research Biologist	LPD NIAID
	R.E. Tarone	Math Statistician	B NCI
	W.B. Jansma	Research Biologist	Dept Path Johns Hopk. Univeristy
	P.M. Steinert	Visiting Scientist	Derm NCI
	J. Pawley	Visiting Scientist	Dept of Zoo. Univ. Wisc.
	H. Ris	Professor	Dept of Zoo. Univ. Wisc.
	S.H. Yuspa	Research Microbiologist	LEP NCI

COOPERATING UNITS (if any)
B, NCI
LEP, NCI
LPD, NIAID

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1	2.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) To discover and develop new methods and interpretive criteria for the application of SEM to a wide variety of soft biological tissues, and thereby capitalize on the unique capabilities of this instrument in providing 1) improved survey and sampling as an adjunct to other studies, 2) an unparalleled three-dimensional, experiential view of cell surface phenomena, and 3) an unusual potential for cytochemical studies.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

The Electron Microscope Laboratory of the Dermatology Branch studies the fine structure and cytochemistry of cells in skin and related tissues to determine their function in normal, hyperplastic and neoplastic states. We are currently working to establish the general significance of cell surface structure and cytochemistry and its relationship to disease processes.

Specifically, we are concerned with: 1) a continued updating of methods used to prepare tissue culture cells for scanning electron microscopy (SEM) with particular emphasis on handling cells in suspension; 2) a continued study of the influences of cell cycle and synchronization procedures on the topography of HeLa cells in culture; 3) a new application of the SEM evaluating the effects of amoscanate on the tegumental surface of Schistosoma mekongi; and 4) a new study visualizing native and reconstituted keratin filaments by means of high voltage electron microscopy (HVEM), and determining the relationship of these cytoskeletal elements to cell surface morphology. The objectives and Approach of the National Cancer Plan that this project most closely supports is 3.5: Study cell surface and cell membrane.

Methods Employed:

1) The current literature has been reviewed to identify published methods used in preparing tissue culture samples for SEM, and the more promising and relevant methods have been evaluated in our laboratory. In conjunction with workshops, tutorials and teaching, we are using this experience to advance both technical standards and standardization in the field and to improve our own routine methods. In particular, the SEM literature on exfoliative cytology samples has been compiled, and representative specimens (courtesy Dr. Gunther Bahr and Ulrika Mikel, AFIP) have been processed by various methods for direct comparison with published results and presented as part of a course on research methods in cytology.

2) To continue our studies of the influence of cell cycle on cultured cell topography, we are examining HeLa cell monolayers grown in unperturbed cultures and parallel samples harvested by mitotic shakeoff and incubated to synchronize cells in the G1, S, and G2 stages of the cell cycle. The cells are exposed to tritiated thymidine for a brief period and fixed. After Feulgen staining, LM photomicrographs are taken of all cells in systematically selected areas of each sample. The samples are then dried and coated with evaporated carbon, and these same cells are micrographed by SEM. The samples are next dipped in liquid emulsion and processed as autoradiographs (ARG), whereupon the same cells are again examined and micrographed either by LM or by SEM in the backscattered electron (BSE) imaging mode. Individual cells are scored as to experiment number, type of specimen (unperturbed culture, synchronization category), stage in the cell cycle (phase of mitosis, labelled or unlabelled interphase), and several morhologic parameters (height and incidence of microvilli, blebs and ruffles),

and these scores compiled in WYLBUR for data processing. This information will assist all investigators examining the topography of unsynchronized monolayer cells to account for the influence of cell cycle. Moreover, there are no previous reports of the impact of synchronization procedures on cell topography. (With Crane, Dvorak, and Tarone).

3) The effects of the first truly successful schistosomicidal drug, amoscanate, on the tegument of the southeast asian strain of blood flukes have been surveyed by SEM. Adult S. mekongi are isolated from mouse portal veins, rinsed and relaxed in physiological saline containing [10^{-6}] M carbachol, and fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for one hour. Specimens are taken from untreated mice and from mice at various times after treatment with a single oral dose (7mg/kg) of amoscanate. The specimens are washed and dehydrated through a graded ethanol series into amyl acetate, dried by the CO2 critical point procedure, coated with evaporated carbon and gold/palladium, and examined with an Etec scanning electron microscope. (With Jansma)

4) In order to study the three dimensional structure of keratin or intermediate filaments and their configuration within keratinocytes, we are utilizing the unique ability of HVEM to visualize these elements within whole spread cells and to resolve fine details (0.3nm) even within thick filament bundles. Reconstituted keratin filaments are prepared by conventional negative staining methods, by embedding and cutting as thick (0.5um) sections, or, after light positive staining with metal salts, dried by the critical point procedure for examination by HVEM and stereoscopic viewing. Cells of primary mouse epithelial cultures are grown under conditions favoring cell spreading on formvar films overlying gold grid supports, then fixed, stained and dried by the critical point method, or embedded and thick-sectioned (0.5um) for examination by HVEM. (With Steinert, Yuspa, Pawley and Ris).

Major Findings:

1) Progress toward optimal, standardized preparative methods for SEM is represented by the following consensus (Workshop on Biological Specimen Preparation, SEM/1981): a) that hypotonic fixative vehicles generally result in cell damage, b) that postfixation in osmium tetroxide is advisable, and c) that shrinkage during the critical point procedure can be minimized by ethanol dehydration into Freon 113 followed by drying in CO2. Also, evaluation of conventional procedures for preparing exfoliated vaginal cells revealed substantial (presumably selective) cell losses from glutaraldehyde-fixed smears (>60%) and from populations settled on poly-L-lysine coated glass coverslips (up to 95%); virtually total retention of these cells was achieved by initially suspending them in fixative, collecting and rinsing them on nucleopore filters, trapping them there in a thin film of distilled water, then quench-freezing, freeze-drying and coating them for SEM observation. These measures will improve specimen quality, interpretation and reproducibility among different labs.

2) Current evidence on the influence of cell cycle on HeLa cell topography confirms previous results from this laboratory obtained with mouse embryo

fibroblasts and chinese hamster ovary (CHO) cells. Unperturbed HeLa cells in culture undergo characteristic surface changes during mitosis, but display little if any topographic change during interphase in association with thymidine incorporation ('S' phase). In the present study we have successfully used atomic number contrast in the BSE mode of the SEM to visualize autoradiographic silver grains that indicate tritiated thymidine incorporation. By scoring topographic features of systemically selected synchronized cell populations and comparing them statistically with unperturbed cell populations, we have preliminary evidence that the simple mitotic shakeoff procedure may alter cell surface form over periods up to 24 hours, independent of progression through the cell cycle. These findings support a first assumption that experimentally induced changes in the topography of cells in monolayer culture do not reflect changes in cycle -- greatly simplifying the interpretation of SEM results in such systems.

3) Progressive surface damage and loss of tegumental integrity was associated with 100% mortality of S. mekongi following a single dose of amoscanate, suggesting that surface damage may underlie the principle mode of action of this drug. Within 6 hours after treatment of the hosts, large blebs, often seen on dying cells, appeared on the dorsal tegument of male schistosomes. More blebs developed on specimens removed at longer periods after drug treatment as the tegument degenerated progressively with outpocketings and erosion of the surface. The tegument of female schistosomes was also affected, but to a lesser extent and with onset as late as 14 days. This application demonstrates the survey capabilities of the SEM to assay drug effects on cell surfaces.

4) These studies are in progress.

Proposed Course of Project:

1) Our standard protocols are being revised in accord with the concensus noted above. Several poorly understood processing steps will be monitored in thin perfusion chambers with Nomarski optics to detect the appearance of artifacts seen by SEM.

2) With this project we are developing the application of data processing techniques to SEM studies of cell surface form. These experiments are being repeated, and combined topographic, Feulgen, thymidine incorporation, and synchronization data are being collected on the same cells. These results will confirm our findings on the influence of cell cycle on monolayer cultures and will elucidate the impact of synchronization procedures on the surfaces of these cells.

3) The nature of the drug-induced surface damage in this system is now being substantiated by transmission electron microscopy (TEM) of samples previously surveyed by SEM and in parallel samples prepared explicity for TEM.

4) We will be comparing chemically defined reconstituted keratin filaments with native intracellular filaments in cultured keratinocytes -- prepared in parallel by several different methods. This approach is designed to verify the intracellular configuration of these cytoskeletal elements, the

substructure of these filaments, the extent to which their appearance depends on preparative methods, and the correspondence of these filaments with topographic details.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03647-08 D

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Scanning and Transmission Electron Microscopy of Leukocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.K. Wetzel	Senior Investigator	Derm NCI
	R.M. Albrecht	Asst Professor Dept of Peds & Pharmacology	Ctr. of Hlth Sci., U. Wisc.

COOPERATING UNITS (if any)

Departments of Pediatrics and Pharmacology, Center for Health Sciences, University of Wisconsin Medical School, Madison, WI.

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.01	0.01	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) To define the surface ultrastructure of leukocytes under a wide range of conditions and thereby provide fresh insight into the physiology of normal and leukemic cells; also, to characterize granules of monocytes, neutrophils, eosinophils, and basophils as an index of the development, function and fate of these cells.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

To define the surface ultrastructure of leukocytes under a wide range of conditions and thereby provide fresh insight into the physiology of normal and leukemic cells; also, to characterize definitively the origin, contents, and fate of the characteristic granules of neutrophils, eosinophils, basophils and monocytes or macrophages as an index of the development, function and fate of these cells. Specifically, in order to establish the functional significance of leukocyte surface features we are initiating a new project extending our studies of cell cycle influences on cell topotopography in mouse embryo fibroblasts, CHO cells and HeLa cells to a similar study in cultured monocytes. We shall correlate the functional parameters of Fc receptor distribution and nonspecific esterase activity with the cell cycle and with topographic configuration nonsynchronized monocyte monolayers.

Methods Employed:

The influence of cell cycle on the topography and function of macrophages is being studied in collaboration with Dr. R. M. Albrecht of the University of Wisconsin. Cells of an established feline macrophage cell line, RAC-1, are grown on glass coverslips or on carbonized formvar films on nickel finder grids. These cells may be a) exposed to tritiated thymidine just before fixation for subsequent autoradiographic identification of S phase, b) fixed briefly and exposed to albumin-antialbumin complexes bound to gold beads for localization of Fc receptors, c) reacted after fixation for cytochemical localization of nonspecific esterase, d) examined by high voltage transmission electron microscopy for cytoskeletal orientation or by scanning electron microscopy for topographic detail, or e) evaluated by any combination of these methods.

We have continued to seek alternative methods for high yield collection, drying, and correlative SEM/LM (light microscopy) of circulating leukocytes fixed directly in peripheral blood.

Major Findings:

Preliminary evidence indicates the suitability of the established macrophage cell line, RAC-1, for further study of the influence of cell cycle on cell topography, ectoenzyme localization and the distribution of surface binding sites. Both ectoenzyme activity and Fc receptor distribution appear related to cell surface features, and possibly to cytoskeletal configuration in these cells. HVEM stereomicroscopy and SEM of these cells, previously examined for nonspecific esterase staining, indicate enzyme localization to ruffled portions of the cell surface with enzyme activity proportional to the numbers of ruffles present. Albumin-antialbumin complexes labelled with colloidal gold also localize to ruffled regions of these cells which are generally devoid of microtubules. These phagocytic macrophages grow vigorously on both glass coverslips and carbonized formvar films such that most cells

spread thinly on the substratum; many of these cells display vigorous non-specific esterase activity and abundant Fc receptors on their surface.

The filtration/freezing procedure developed in this laboratory remains the method of choice, although it is limited by the extremely low incdence of leukocytes in peripheral blood.

Significance to Cancer Research:

The reactions of lymphocytes and macrophages to immune responses in malignant growth and therapy are largely dependent on cell surface phenomena and cell interaction -- both uniquely amenable to study by SEM and ancillary methods.

A thorough understanding of the origin and nature of leukocyte granules is necessary to define and interpret the differentiation of normal granulocytes, the cytopathology of chronic granulocyte leukemia and related disorders, and to help clarify the nature and functions of granulocytic infiltrates in skin and other diseases in malignant diseases.

Proposed Course:

Effects of the cell cycle on ectoenzyme and Fc receptor distribution, and on cell topography and cytoskeletal configuration will be correlated in the macrophage cell line RAC-1. Experience with other cell types indicates that the morphological parameters are substantially affected by mitosis, if not by interphase stages of the cell cycle, and this system offers a unique opportunity to correlate funtional parameters as well. Once these relationships are established these cells can be compared with circulating monocytes and with peritoneal macrophages activated by various means.

To improve the effectiveness and reliability of poly-L-lysine for attaching fixed cells from suspension, we are exploring the use of added ethanolamine, prior periodate oxidation, and the use of flourescamine to monitor the distribution of poly-L-lysine on the substrate. Pending improvement of our methods for retaining cells from suspension we will be engaged in a thorough description of the topography of peripheral leukocytes from normal humans obtained in representative yields. This approach will be extended to studies of hematopoiesis and the leukemias. A major study of leukocyte granulogenesis is planned employing quantitative autoradiography, cytochemistry and TEM. Light microscopie monitoring of preparative conditions and the dynamics of leukocyte adherence, motility, interaction, and growth will be continued with the use of thin perfusion chambers.

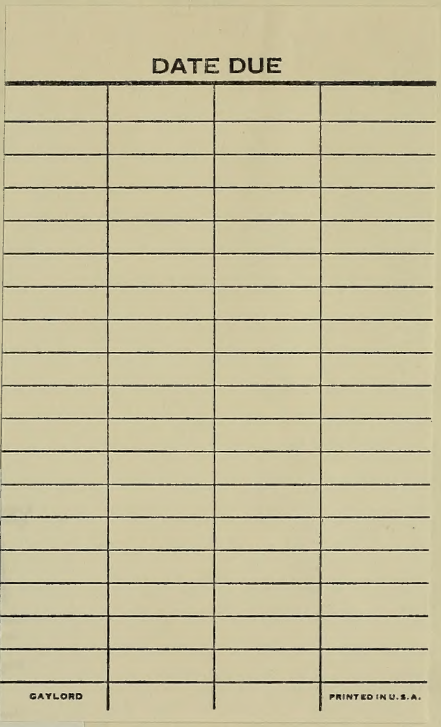
Publications:

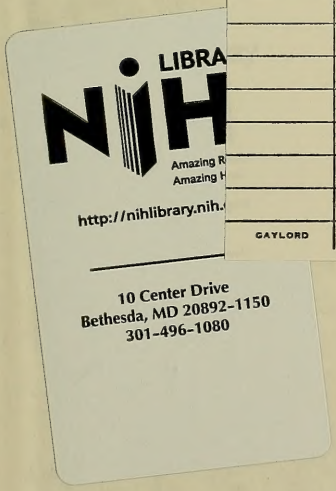
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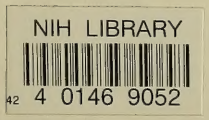
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